MASTER (MA)

Medicinal chemistry of aminoglycosides

Al Khzem, Abdulaziz

Award date:
2019

Awarding institution:
University of Bath

Medicinal chemistry of aminoglycosides

Abdulaziz Hassan Alkhzem

A thesis submitted for the degree of Master of Philosophy

University of Bath

Department of Pharmacy and Pharmacology

December 2018

Signed ……

Date …….

ABSTRACT

It is demonstrated that NMR spectroscopy is a powerful technique for separating and measuring each distinct pK_a value of the amino groups located around aminoglycoside antibiotics. Unambiguous assignments were made for each individual amine and guanidine substituent on: 2-deoxystreptamine, neamine, neomycin C, paromomycin, tobramycin, kanamycin B, netilmicin, sisomicin, amikacin, and streptomycin using variations in the NMR spectroscopic chemical shift (δ) with ^{1}H, ^{13}C, and ^{15}N HMBC as the reporter probes. These data were then compared with the literature data. Minor revisions to the assignment order were made for neomycin C and paromomycin. pK_a values for kanamycin B have been reported using potentiometry, however no NMR derived pK_a data have been reported, and for netilmicin no literature pK_a values were found. Therefore, these data are reported for the first time herein.

Due to its sensitivity and natural abundance (99.98%), ^{1}H NMR is less time consuming than ^{13}C and ^{15}N HMBC NMR spectroscopy. For this reason, ^{1}H NMR is the most common and preferable method for measuring individual pK_a values.

One of the ultimate objectives of this line of research is to be able to trace an aminoglycoside, e.g., tobramycin, in the body in a non-invasive way by tagging the aminoglycoside with a fluorescent probe. Taking both the pK_a values of the individual amino functional groups of tobramycin and steric-hindrance factors into consideration, the investigation of the specific or selective reactions of the different amines located around tobramycin were carried out with amino acid protecting groups, e.g., Boc and Cbz, and with a fluorophore (FITC) that would generate a biologically relevant tagged product. Four out of five amines on tobramycin were protected with Boc protecting groups. However, the mono-protection of tobramycin with Boc or Cbz groups and labelling the unprotected (unreacted) N-3 amine on tetra-Boc protected tobramycin with FITC were not successful.

DEDICATION

for

Roqia Moudis Alzhrani

Hassan Awad Alkhzem

Sundus Jubran Alqahtani

ACKNOWLEDGEMENTS

I gratefully thank my parents and my family for their unlimited support all the way. Nothing could be achieved without their constant encouragement.

I sincerely thank my supervisors Dr Ian S. Blagbrough and Dr Timothy J. Woodman for their advice, guidance, understanding, kind encouragement, and continuous support throughout my MPhil.

I sincerely thank the Government of the Kingdom of Saudi Arabia for fully funding this studentship. I also thank the Departmental of Pharmaceutical Chemistry within the College of Clinical Pharmacy at Imam Abdulrahman Bin Faisal University, Dammam, KSA, for their encouragement and support.

I thank all the technical staff in the Department of Pharmacy and Pharmacology, University of Bath, for all their assistance. I also thank all my laboratory colleagues in 5W3.14 for their help and for the friendly working environment. I thank all the students in the office for fun chats that certainly made my time in Bath enjoyable.

ABBREVIATIONS

d	Doublet
equiv.	Equivalent
ESI	Electrospray ionisation
FITC	Fluorescein isothiocyanate
g	gram
HMBC	Heteronuclear multiple bond correlation
HR TOF	High resolution time-of-flight
HRMS	High resolution mass spectrometry
HSQC	Heteronuclear single quantum correlation
Hz	Hertz
M	Molar (moles per litre)
m	Multiplet
mmol	Millimoles
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser spectroscopy
pK_a	Ionisation constant of the conjugate acid
ppm	Parts per million
q	Quartet
R_f	Retention factor
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
s	Singlet
t	Triplet
TLC	Thin layer chromatography
UV	Ultraviolet
v/v	Volume/volume
WHO	World Health Organisation

CONTENTS Page number

Chapter 1

General Introduction to Aminoglycosides

1.1. Aminoglycosides

1.1.1. General Introduction to Aminoglycoside Antibiotics

By definition, natural products are any product that has been isolated or produced from microorganisms, animals or plants present in nature (Schoental, 1965; Gad, 2005; Armstrong et al., 2012). According to the World Health Organization (WHO), more than 80% of the world's population relies on natural products through traditional or alternative medicines for their healthcare (Farnsworth et al., 1985). More than 100 of the chemical substances currently in use have been isolated from natural sources such as plants and/or microorganisms. One example of such a natural products class are the aminoglycosides (Cragg and Newman, 2001; Gad, 2005).

Aminoglycosides are compounds which consist of an aminocyclitol moiety 2-deoxystreptamine (1) or streptidine ring in streptomycin (10) (see Fig. 1.1 and Fig. 1.3) attached to amino sugars by glycosidic bonds. These compounds are primarily used for the treatment of infection by Gram-negative (aerobic) and other Gram-positive bacteria (Seiler et al., 1996; Foye, et al., 2008; Beale et al., 2010; Ramirez and Tolmasky, 2010; Watkins et al., 2013).

1.1.2. History of Aminoglycoside Antibiotics

There are many drug subtypes in aminoglycosides, including: neomycin C (3), paromomycin (4), tobramycin (5), kanamycin B (6), netilmicin (7), sisomicin (8), amikacin (9), and streptomycin (10), (see Fig. 1.1, 1.2, and 1.3). They occur both naturally and in the form of semi-synthetic compounds (Weisblum and Davies, 1968; Shah et al., 1977; Whelton and Neu, 1982; Hofer, 2013). These antimicrobial agents were first discovered in 1944 from the organism *Streptomyces griseus*. Streptomycin (10) and neomycin (3) were revealed by American biochemists Selman Waksman, Elizabeth Bugie, and Albert Schatz from bacteria in the soil (Schatz et al., 1944; Beale et al., 2010; Armstrong et al., 2012).

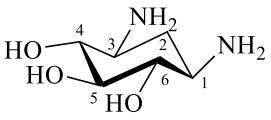
The first drug in the aminoglycoside series was streptomycin (10). It is efficacious in the treatment of tuberculosis (Tb) which was previously a life-threatening condition. New members in the aminoglycoside series, e.g., kanamycin B (6) and tobramycin (5), continue to contribute to healthcare. In the 1970s, other agents, namely dibekacin, amikacin, and netilmicin (7), were also introduced; these are semi-synthetic aminoglycosides (Avent et al., 2011).

Aminoglycoside antibiotics comprising of neomycin (3), introduced in 1949, paromomycin (4), introduced in 1956, kanamycin B (6), introduced in 1957, gentamicin (11), introduced in 1963, tobramycin (5), introduced in 1967, and sisomicin (8), introduced in 1970, and (Table 1.1). All of these drugs have shown great potential for halting the activity of Gram-negative bacteria, such as *Pseudomonas aeruginosa* and some Gram-positive bacteria, such as *Staphylococcus aureus* (Schatz et al., 1944; Waksman and Lechevalier, 1949; Armstrong et al., 2012; Becker and Cooper, 2013). New semi-synthetic drugs were introduced because bacteria were becoming resistant to the previous aminoglycosides. A situation which, if anything, is even worse today with the rise of superbugs and antimicrobial resistance on a global scale. Antimicrobial resistance has been caused by the poor stewardship of antibiotics and we are at risk of entering the post-antibiotic era. Therefore, a more detailed, scientific understanding of antibiotics will help in the discovery of more and potentially better

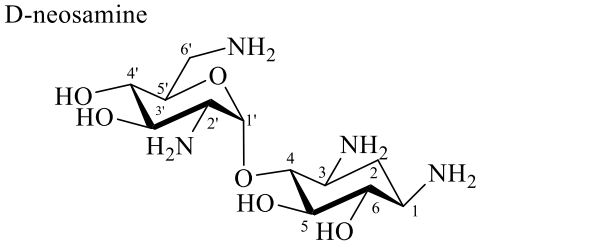
antibiotics. The new semi-synthetic drugs had better pharmacological profiles than the previous aminoglycosides and were called the second generation of aminoglycosides. These included the following: dibekacin, introduced in 1971, amikacin, introduced in 1972, arbekacin, discovered in 1973, isepamicin, discovered in 1975, and netilmicin (7), discovered in 1976. It was also found that the newly developed semi-synthetic aminoglycosides, especially amikacin, were less susceptible to targeting by aminoglycoside-modifying enzymes (AMEs) (Becker and Cooper, 2013).

Table 1.1 Year of discovery, source, and the spectrum of activity of selected aminoglycosides

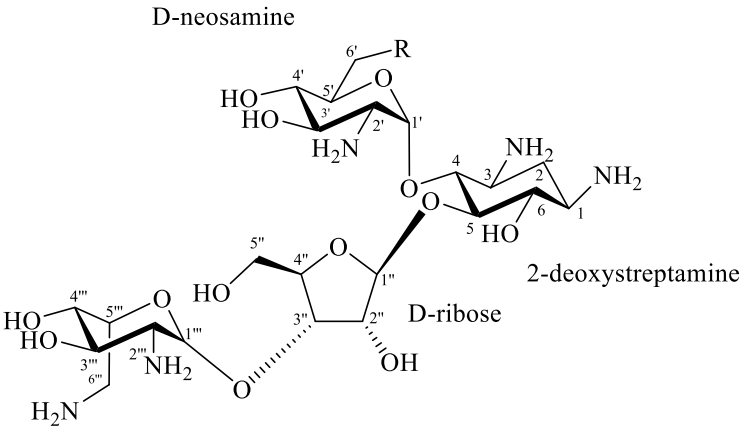
Aminoglycoside	**Discovery**	**Source**	**Spectrum of activity**
streptomycin (10)	1944	*Streptomyces griseus*	*Mycobacterium tuberculosis*
neomycin (3)	1949	*Streptomyces fradiae*	*Enterobacteriaceae spp*
paromomycin (4)	1956	*Streptomyces rimosus*as	*Protozoa*
kanamycin B (6)	1957	*Streptomyces kanamyceticus*	*Enterobacteriaceae spp*
gentamicin (11)	1963	*Micromonospora purpurea*	*Pseudomonas*
tobramycin (5)	1967	*Streptomyces tenebrarius*	*Pseudomonas*
sisomicin (8)	1970	semi-synthetically from gentamicin	*Enterobacteriaceae spp* *Pseudomonas aeruginosa*
amikacin (9)	1972	semi-synthetically from kanamycin	*Enterobacteriaceae spp* *Pseudomonas aeruginosa*
netilmicin (7)	1976	semi-synthetically from gentamicin	*Enterobacteriaceae spp* *Pseudomonas aeruginosa*



2-deoxystreptamine (1)



neamine (2)



neomycin (3) R = NH_2
paromomycin (4) R = OH

Fig. 1.1 2-Deoxystreptamine (1), neamine (2), neomycin C (3), paromomycin (4)

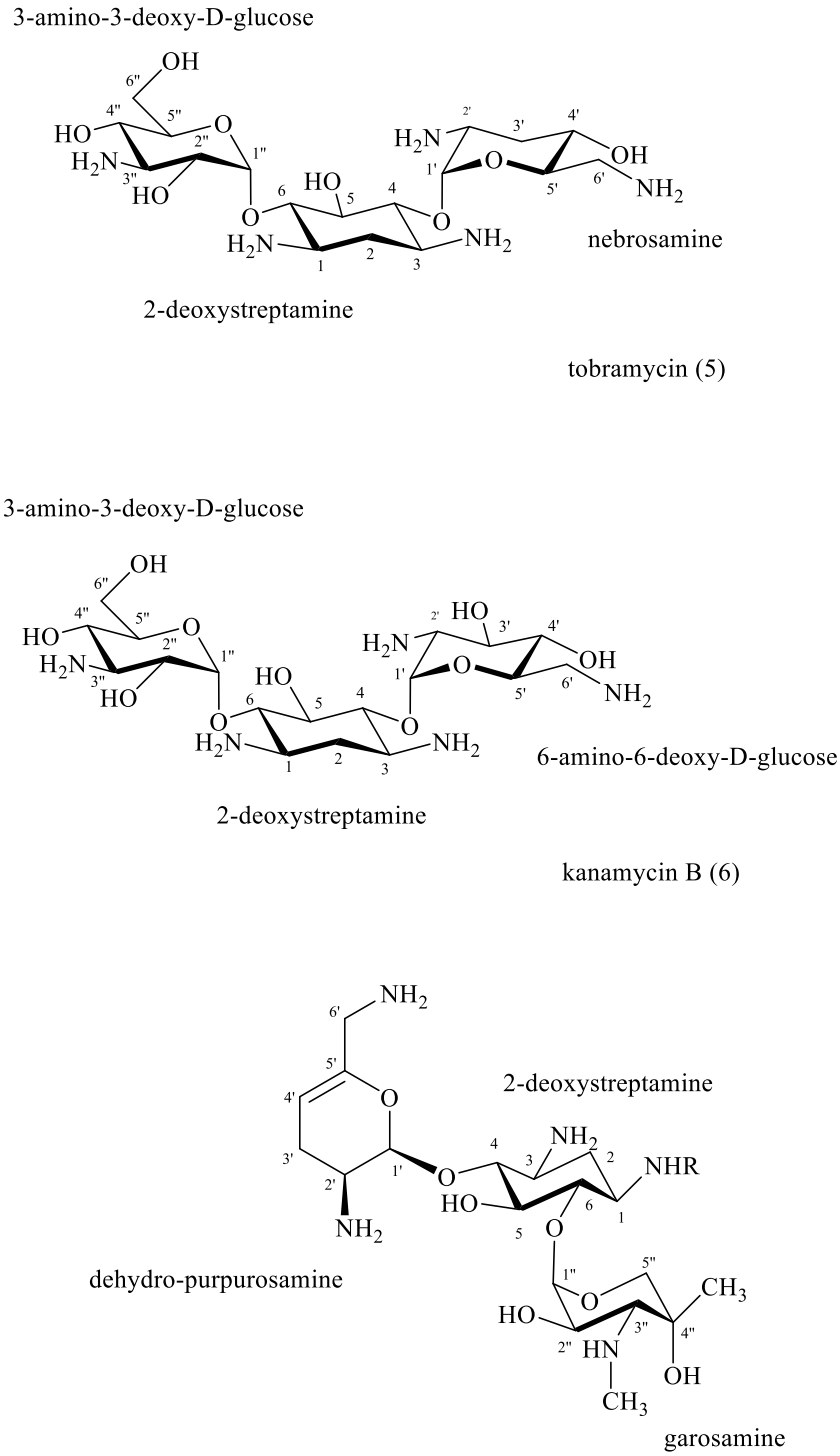
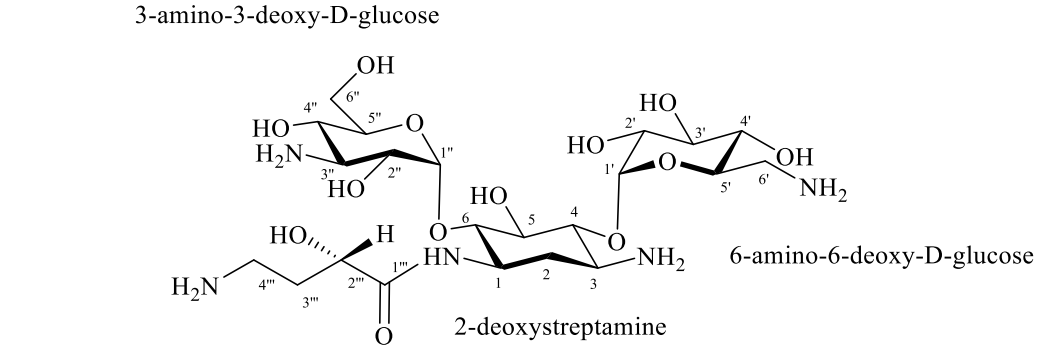
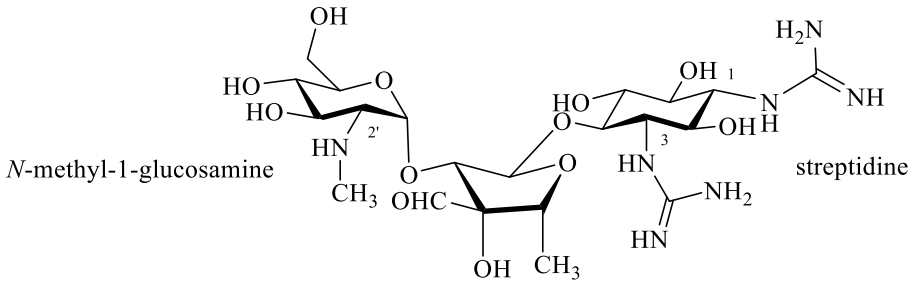


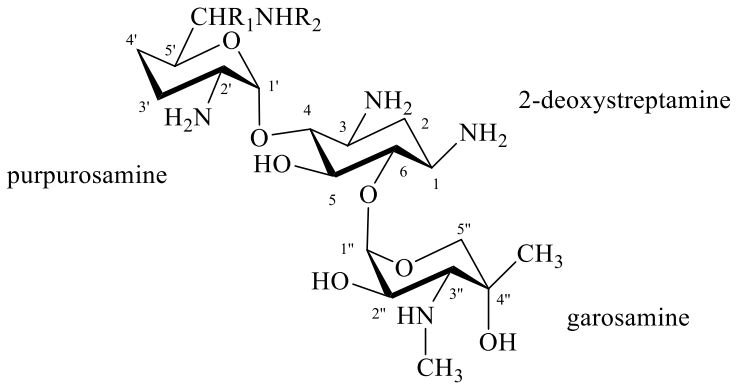
Fig. 1.2 Tobramycin (5), kanamycin B (6), netilmicin (7), sisomicin (8)



amikacin (9)



streptomycin (10)



gentamicin (11) C1: $R_1 = R_2 = CH_3$
C1a: $R_1 = R_2 = H$
C2: $R_1 = CH_3 = R_2 = H$

Fig. 1.3 Amikacin (9), streptomycin (10), gentamicin (11).

1.1.3. Mechanism of Action of Aminoglycosides

The mechanism of action of aminoglycosides allows them to attack the aerobic, Gram-negative bacteria (Foye et al., 2008; Beale et al., 2010; Becker and Cooper, 2013; Watkins et al., 2013). The mechanism was identified in 1980, where it was found that they act on the 16S rRNA subunit of the 30S bacterial ribosome (Moazed and Noller, 1987; Kong et al., 2016). Since then, there has been extensive research to identify the various methods with which aminoglycosides attack bacteria. The general mechanism of the action of aminoglycosides resides in their ability to displace adenine from the three unpaired adenine residues in the decoding loops. This causes an orientation into a flipped-out position and consequentially, the development of non-functional protein synthesis, leading to cell death, shown in Fig. 1.4 (Vicens and Westhof, 2003; Ogle and Ramakrishnan, 2005; Kulik et al., 2015).

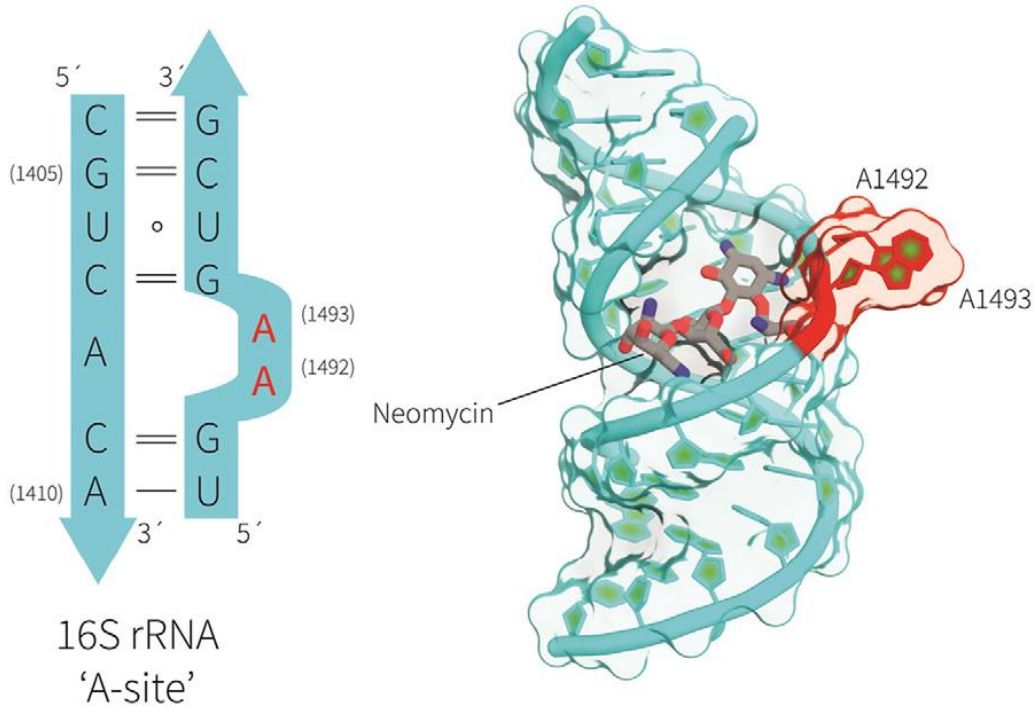


Fig. 1.4 Neomycin B binds to 16S RNA ribosomes at the A-site of the 30S ribosomal subunit (taken from Kong et al., 2016)

The binding of aminoglycosides to RNA is associated with the interaction of the amino groups of aminoglycosides, which are positively charged, and the anionic phosphate groups of RNA (Wang and Tor, 1997). The other source of interaction occurs because of the hydrogen bonding between different hydroxyl and amino groups of aminoglycosides and RNA (Jiang and Patel, 1998; François et al., 2005; Hobbie et al., 2006). All of these interactions result in a bond between RNA and aminoglycosides which results in a decline in the translation activity of bacterial RNA.

The introduction of L-4-amino-2-hydroxybutanoic acid (L-AHBA) on the N-1-position of the 2-deoxystreptamine ring on amikacin (9) (see Fig. 1.3) was found to be efficacious in improving the attacking profile of aminoglycosides on the bacteria. Adding L-AHBA can make a stronger bond with the A-site of the RNA of the bacteria. Moreover, a primary amine present in the L-AHBA on amikacin, another domain for the interaction with the phosphate of RNA of bacteria, results in strong bonding (Russell et al., 2003). An analogue of tobramycin (5) has been introduced that has shown great efficacy against bacteria that produce aminoglycoside-modifying enzymes. 6″-Thioether-tobramycin attacks the bacterial cell membranes rather than the protein synthesis process (Herzog et al., 2012). An important aspect for the clinical use of aminoglycosides is their potential use in human immunodeficiency virus (HIV) treatment as they can target many important sites in the HIV life cycle (Houghton et al., 2010). This has led to a developing interest among researchers to look for ways to treat patients with HIV using aminoglycosides. Aminoglycoside–polyarginine conjugates (APArC) and aminoglycoside–arginine conjugates (AArC) are some of the compounds under consideration for the treatment of HIV (Hegde et al., 2007; Lapidot et al., 2008; Mousseau et al., 2015).

1.1.4. Spectrum of Activity of Aminoglycosides

The spectrum of activity means the ability of the antibiotic to target organisms, specifically it is defined as the efficacy of the drug to target certain organisms (Armstrong et al., 2012; Carpenter et al., 2012). Narrow-spectrum antibiotics include streptomycin (10). These are

effective against Gram-negative organisms, such as *Salmonella* species, *Pseudomonas aeruginosa*, *Escherichia coli*, *Pasteurella* species and *Brucella* species (see Table 1.2) (Whelton and Nue, 1982).

Neomycin has the ability to target Gram-negative bacteria and also exhibits activity against Gram-positive bacteria. It is usually used orally before gastrointestinal surgery and to reduce the risk of infection after intestinal surgeries. Neomycin B is found in ointments and used topically to treat bacterial skin diseases (Heidary and Cohen, 2005; Joint Formulary Committee, 2018).

Gentamicin (11) is the aminoglycoside of choice in the United Kingdom (UK) (Joint Formulary Committee, 2018). It has activity against many Gram-negative bacteria (see Table 1.2) (Whelton and Nue, 1982). It has its lowest minimum inhibitory concentration (MIC) against *Staphylococcus epidermidis* (MIC = 0.19), while its highest MIC is against *Pseudomonas aeruginosa* (Lebeaux et al., 2015). Gentamicin (11) is also effective in the treatment of pneumonia and urinary tract infections (UTIs) (Foye et al., 2008; Balakumar et al., 2010; Armstrong et al., 2012).

Amikacin (9) is another aminoglycoside which exhibits a spectrum of activity against many organisms. It is effective against *Pseudomonas aeruginosa*, *Escherichia coli*, *Shigella* sp., *Salmonella* sp. and *E. cloacae*, as illustrated in Table 1.2 (Whelton and Nue, 1982). Amikacin (9) is used in the treatment for some strains of *Mycobacterium tuberculosis* and *Yersinia tularensis* infections that are resistant to other aminoglycosides (Joint Formulary Committee, 2018). Amikacin (9) has the highest MIC against *Pseudomonas* and has the lowest MIC against *Klebsiella pneumonia* (Balakumar et al., 2010; Armstrong et al., 2012).

Tobramycin (5) is one of the new aminoglycosides with new and improved activity. It is also a broad-spectrum antibiotic. Tobramycin (5) is more effective against *Pseudomonas aeruginosa* and is even more efficacious in the treatment of strains, which are resistant to gentamicin (11), as shown in Table 1.2 (Whelton and Nue, 1982). However, tobramycin (5) is not efficacious against gentamicin-resistant *Enterobacteriaceae* (LoBue, 2005; Zeitler et al., 2012; Joint Formulary Committee, 2018). A tobramycin (5) plus carbenicillin combination

therapy is effective in the treatment of endocarditis caused by *Pseudomonas* which shows resistance to gentamicin (11) plus carbenicillin (Herzog et al., 2012). The most common or frequently prescribed aminoglycosides in the Kingdom of Saudi Arabia (KSA) are tobramycin (5), amikacin (9), and gentamicin (11) (Asghar and Ahmad, 2018).

Kanamycin (6) has been used in the treatment of bacterial infectious for more than 60 years (Zhang et al., 2008). Kanamycin (6) has an efficacy against *Enterobacter* and *Pseudomonas* species, as indicated in Table 1.2 (Whelton and Nue, 1982; Bera et al., 2010; Ahmad and Mokaddas, 2014). Paromomycin (4) is efficacious against *Cryptosporidium*, *Entamoeba* and *Giardia*. Paromomycin (4) is used to treat different kinds of infections such as, giardiasis and amebiasis. Paromomycin (4) is the first line therapy for giardiasis and amebiasis during the pregnancy (Beale et al., 2010). Netilmicin (7) and sisomicin (8) have the same activity as tobramycin (5) and amikacin (9) (Beale et al., 2010).

Table 1.2 Vulnerability of some strains of bacteria to some of aminoglycosides at Massachusetts General Hospital from the 1st of January to 25th of December in 1978 (Whelton and Nue, 1982)

Organism	**Percentage susceptible to streptomycin**	**Percentage susceptible to gentamicin**	**Percentage susceptible to amikacin**	**Percentage susceptible to tobramycin**	**Percentage susceptible to kanamycin**
Pseudomonas aeruginosa	5 %	81 %	81 %	96 %	4 %
Proteus mirabilis	87 %	97 %	97 %	98 %	92 %
Escherichia coli	64 %	98 %	98 %	98 %	85 %
Shigella sp.	33 %	100 %	100 %	100 %	96 %
Salmonella sp.	52 %	100 %	98 %	98 %	98 %
Klebsiella pneumoniae	81 %	87 %	99 %	87 %	83 %

The Food and Drug Administration (FDA) Antimicrobial Drugs Advisory Committee (ADAC) has recently (May 2018) voted in favour of plazomicin (Achaogen) for the treatment of adults with complicated urinary tract infections (cUTI) due to MDR Enterobacteriaceae including carbapenem-resistant Enterobacteriaceae (CRE), e.g. Klebsiella pneumoniae carbapenemase (KPC), New Delhi metallo-β-lactamase (NDM) (FDA, 2018).

1.1.5. Chemistry and Structure-Activity Relationship (SAR) of Aminoglycosides

Aminoglycosides can be divided into multiple subtypes and classes depending on their arrangement and synthesis. Aminoglycosides contain a 1,3-diaminoinositol moiety composed of either 2 deoxystreptamine ring (1), as in neamine (2), neomycin (3), paromomycin (4), tobramycin (5), kanamycin (6), netilmicin (7), sisomicin (8), and amikacin (9) or a streptidine ring, as in streptomycin (10) linked to inositol derivatives, such as L-neosamine, D-neosamine, purpurosamine, garosamine, D-ribose, and nebrosamine by glycosidic bonds. In fact, the name aminoglycoside inspired from its structure of aminosugars linked by glycosidic bonds (see Fig. 1.1, 1.2, and 1.3) (Maviglia etal., 2009; Beale et al., 2010; Avent et al., 2011; Rahim et al., 2011; Hooper, 1982).

Amino groups on the D-neosamine ring in neomycin (3), nebrosamine in tobramycin (5), purpurosamine in netilmicin (7) and sisomicin (8) are important for the antibacterial activity (see Fig. 1.1 and 1.2) (Benveniste and Davies, 2016). The main culprit in the development of resistance to these aminoglycosides was related to aminoglycoside-modifying enzymes (AMEs) that are produced by bacteria. These enzymes are associated with changes in the −OH or $-NH_2$ groups on aminoglycosides, catalysed by enzymes such as *N*-acetyltransferase (NAT), *O*-adenyl transferase (OAT) and *O*-phosphoryl transferase (OPT), as shown in Fig. 1.5 (Mingeot-Leclercq et al., 1999: Kling et al., 2007; Foye et al., 2008; Beale et al., 2010; Houghton et al., 2010). Amino groups at positions 2' and 6' of neomycin C (3), paromomycin (4), and sisomicin (8) are crucial for antibacterial activity (Beale et al., 2010). Some chemical changes on 2-deoxystreptamine's functional groups are possible without any loss of

antibacterial activity. For example, amikacin (9) results from the acetylation of the N-1 of kanamycin (Fig. 1.6).

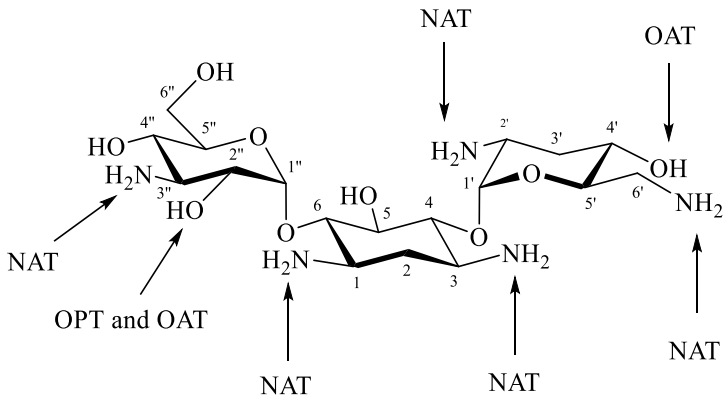


Fig. 1.5 The modifications of bacterial enzymes on tobramycin

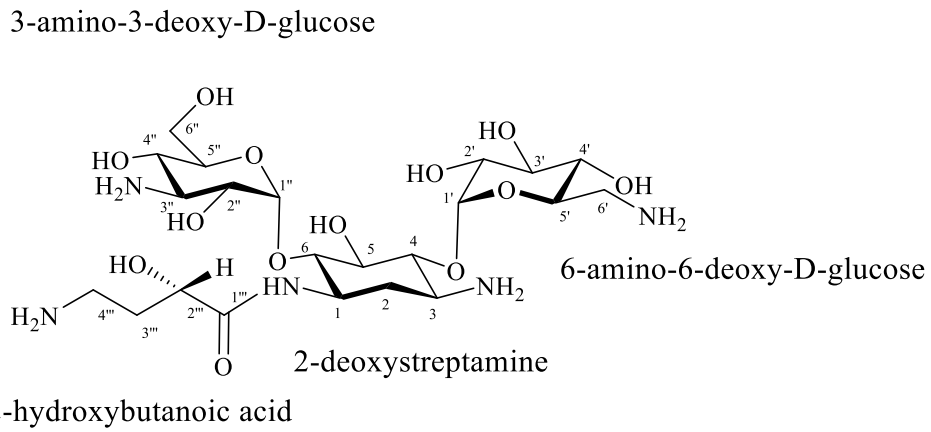


Fig. 1.6 Amikacin results from acetylation of the N-1 of kanamycin

Tobramycin (5) is one of the broad-spectrum aminoglycosides. One of its analogues is the 6″-thioether. This analogue is amphiphilic and has activity against tobramycin-resistant bacteria, as it is less prone to be activated by bacterial aminoglycoside-modifying enzymes. The lipophilic group of this analogue is effective due to its potency against bacteria and selective nature towards the bacterial membranes (Herzog et al., 2012).

The addition of 4', 6'-*O*-acetal and 4'-*O*-ether on the 2-amino-2-deoxy-D-D-glucose ring of paromomycin (4) has the ability to enhance the selectivity of paromomycin, allowing it to target only the bacterial ribosome (Perez-Fernandez et al., 2014).

1.1.6. Side Effects of Aminoglycosides

Aminoglycosides are drugs that are notorious for their side effects, which are associated with kidneys and hearing systems. The mechanisms of ototoxicity and nephrotoxicity of aminoglycoside antibiotics are incompletely understood. There are certain factors which are associated with the development of toxicity or side effects in patients taking aminoglycosides, including genetic pre-disposition, use of concomitant drugs, pre-existing disease and severity of illness. Prolonged use of aminoglycosides is also associated with toxicity (Avent et al., 2011).

The ototoxicity of aminoglycosides is divided into two types, namely vestibular toxicity and cochlear toxicity. The ototoxicity of these antibiotics is associated with their ability to penetrate the inner ear via the bloodstream where it then affects the cochlea or the vestibular system (Foye et al., 2008; Avent et al., 2011; Armstrong et al., 2012). One possible mechanism for ototoxicity is that aminoglycosides cause the modulation of the *N*-methyl-D-aspartate receptors (NMDAR) a subtype of L-Glutamate receptors (GluR). Glutamate is an important neurotransmitter in both the hair cells and the inner ear. Excessive glutamate has been linked with CNS toxicity and ear toxicity. Moreover, aminoglycosides are possibly related to high levels of nitric oxide that causes a release of free radicals. These free radicals are associated with damage to the proteins and other targets of the outer hair cells (OHCs) of the cochlea and the supporting cells of the basal region of the cochlea (Ricci, 2008; Armstrong et al., 2012; Petersen and Rogers, 2015). Ototoxicity developed through the use of aminoglycosides is irreversible (Selimoglu, 2007; Armstrong et al., 2012; Petersen and Rogers, 2015).

Aminoglycoside antibiotics are nephrotoxic agents (Mingeot-Leclercq and Tulkens, 1999; Armstrong et al., 2012; Brenner and Stevens, 2013). They are excreted by the kidneys via glomerular filtration, and they might have a tendency to accumulate in the kidney cortex. This leads to a decline in the lysosomal phospholipase activity causing aminoglycoside-induced nephrotoxicity. The increase in number and size of lysosomes have been found with decreased lysosome stability. Another possible mechanism for nephrotoxicity is that aminoglycosides

may cause ultrastructural damage to the tubular epithelium or to the endothelium of the glomerular capillaries, however, this toxicity can be reversed with good hydration (Cojocel and Hook, 1983; Janknegt, 1990; Houghton et al., 2010; Armstrong et al., 2012).

One of the lesser toxicities caused by aminoglycosides is neuromuscular toxicity. It is related to blockade caused by the aminoglycosides at the synaptic level. This neurotoxicity is associated with the use of certain drugs that block the neuromuscular junction (NMJ) whereby the presence of pre-existing illness combines to worsen the condition, such as myasthenia gravis (Burton, 2006; Avent et al., 2011).

As a standard precaution, it is necessary for patients receiving aminoglycosides to be given notice regarding their toxicity. They should be able to identify and report any incidence of tinnitus, change in hearing, hearing loss, and vertigo (Burton, 2006; Avent et al., 2011). Monitoring the toxicity is also an important aspect in the treatment with aminoglycosides. There are no standards that could aid in the prevention, diagnosis, and treatment of toxicity, but certain tests should be carried out as a precaution. These can include quantitative testing of end-organ results (monitoring serum creatinine and audiometry) (Burton, 2006; Houghton et al., 2010).

The use of aminoglycosides has decreased due to their side effects and the controversial debate regarding their mechanisms of toxicity. In order to understand their mechanisms of toxicity, one of the ultimate objectives is to be able to trace these natural compounds in the body in a non-invasive way by tagging them with a fluorescent probe. The use of fluorescent derivatives of aminoglycosides may provide an insight to understand their mechanisms of toxicity. However, random FITC-labelling may present some obstacles in which it can prevent the effectiveness of aminoglycosides by blocking their biological activity. Therefore, this study aims to investigate the specific or selective reactions of different amines located around tobramycin using FITC, depending on very important factors in medicinal chemistry, which are steric effects and the ionisation constants (pK_a) of these amines.

1.2. Ionisation Constant

1.2.1. General Introduction to Ionisation Constant (pK_a)

Ionisation constants (pK_a of conjugate acid) are among the most frequently studied parameters and give considerable information about the physical and kinetic behaviour of chemical substances. pK_a is defined as the pH at which functional groups, such as amino groups and carboxylic groups, exist as 50% ionized and 50% un-ionized. The first concept of the dissociation constant comes from the law of mass action, provided by Guldberg and Waage (1864). The concept was further strengthened by the works of Henderson and Hasselbalch (Henderson, 1908; Hasselbalch, 1916). pK_a is expressed as the following:

$$pK_a = - \log K_a$$

In this equation, K_a is dissociation constant of the reaction. The dissociation of an acid in a solvent, such as water, is a reversible process proceeding in both forwards and backwards directions; the more dissociation, the stronger an acid is. The extent of the dissociation is measured in terms of K_a (Henderson, 1908; Hasselbalch, 1916; Po and Senozan, 2001; De Levie, 2003).

$$K_a = [H_3O^+] [A^-] / [HA]$$

K_a is the ratio of dissociated and un-dissociated acid in a solution and thus measures the strength of an acid in a solution. The higher K_a, the stronger the acid is because of the higher dissociation, in other words, the lower the pK_a, the stronger an acid, as shown in Fig. 1.7 (Albert and Serjeant, 1984). The main advantage of using pK_a instead of K_a is convenience. Due to the use of a logarithmic scale, a very large range of concentrations is covered in a convenient way. Use of this scale also has the advantage of converting very small values into easily tangible digits.

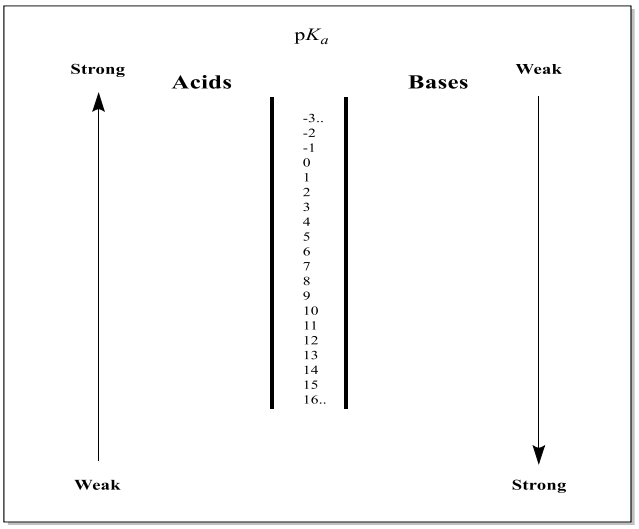


Fig. 1.7 The lower the pK_a, the stronger an acid, the higher the pK_a, the weaker an acid.

Although the ionisation constants are considered to be constant, they are affected by some factors. The ionisation constants differ with changes in the length of the alkyl chain between two functional groups. One reason for this change is the inductive effect. When the distance between two functional groups increases the inductive effect decreases and vice versa (see Fig. 1.8). The steric and resonance effects might decrease the pK_a values of the functional groups, as shown in Fig. 1.8 (Takeda et al., 1983; Albert and Sargeant, 1984; Blagbrough et al., 2011). Takeda et al., (1983) used ^{15}N NMR spectroscopy to determine the pK_a values of spermidine.

Examples for presenting ionisation constants of each ionisable group are cadaverine (1.5-diaminopentane) and the closely related amino acid lysine. There are two ionisable groups in cadaverine, namely the two primary amines (NH_2), whereas there are three ionisable groups in lysine, namely the carboxylic acid and the two similarly spaces amines groups. The pK_a values of each ionisable group and the balanced net ionic equations for both compounds are shown in Fig. 1.9 (Zhang and Vogel, 1993).

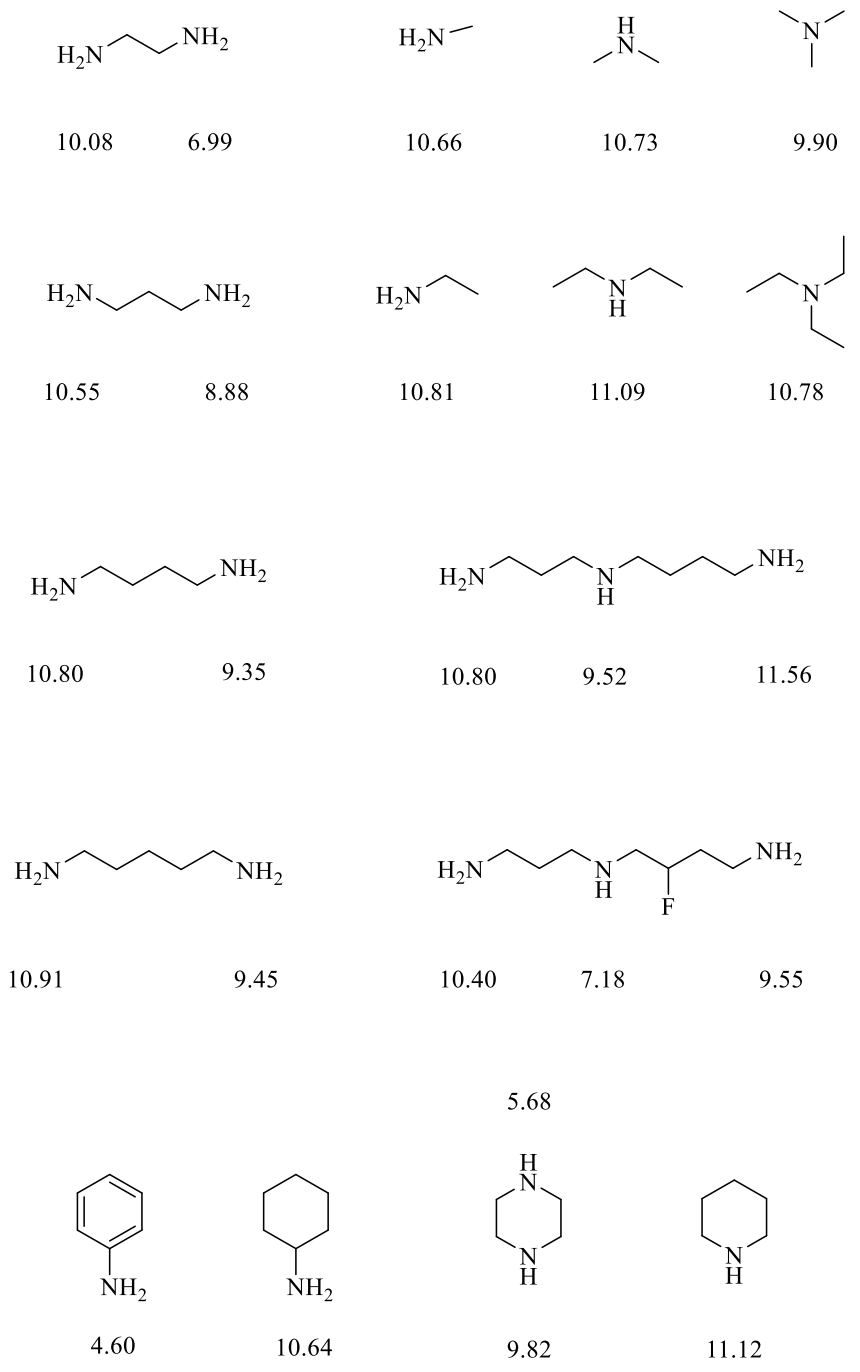
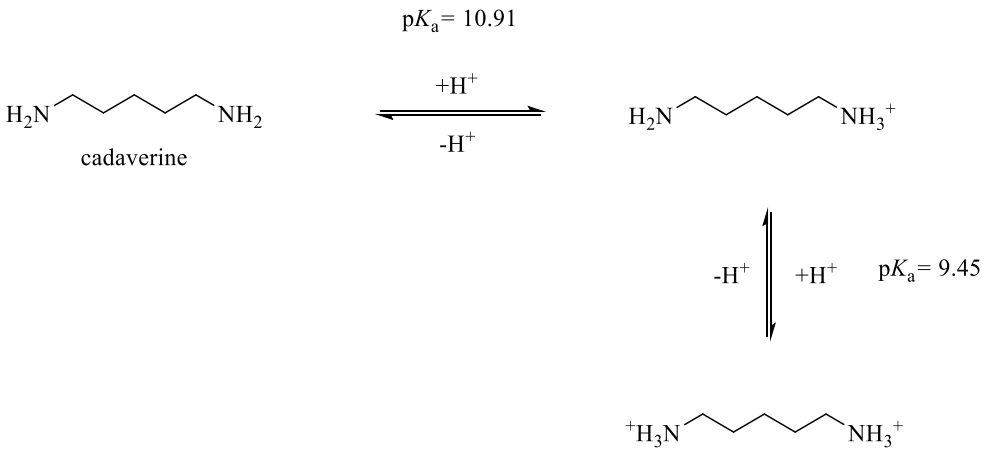


Fig. 1.8 The increases of the alkyl chain distance lead to an increase in the pK_a values of amines, the steric and resonance effects might decrease the pK_a values of the amino groups in H_2O (Takeda et al., 1983; Albert and Sargeant, 1984; Blagbrough et al., 2011)

A)



B)

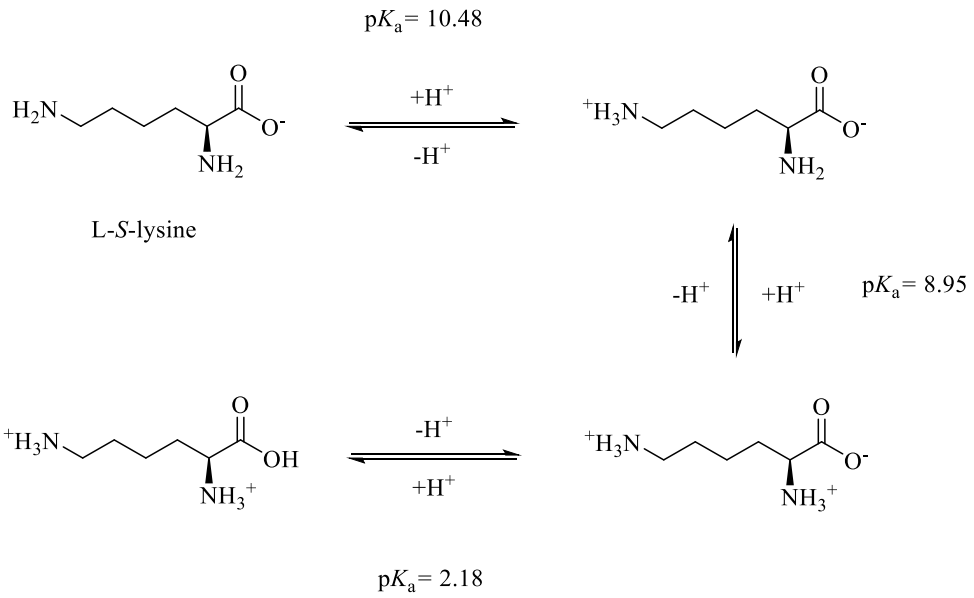


Fig. 1.9 The balanced net ionic equations of cadaverine (A) and L-*S*-lysine (B) for the reaction corresponding to each pK_a in H_2O

Measuring the ionisation constants of any medication is significant in a variety of fields, such as food science, organic chemistry, analytical chemistry, drug development, and drug discovery (Bezençon et al., 2014). The importance of the knowledge of the dissociation constants of drugs and other substances is considered high in pharmaceutical systems as it

yields valuable information. Such information is important mainly because many drugs are either weak acids or weak bases. The knowledge of the ionisation constants gives information about the ionic properties of the drugs when exposed to a variety of pH range inside the body. When a drug is released from an orally ingested medicine, it is first exposed to the extremely acidic pH of the stomach and then the basic pH along the intestines. For drugs that are ionized at the physiological pH of the digestive system, there exists a requirement of specific transporters for the transportation across the epithelium of the digestive-(gastro-intestinal) tract. The drugs that are unionized can be absorbed by simple passive diffusion. Similarly, when absorbed into the bloodstream, drugs are exposed to an almost neutral or slightly basic pH of 7.4. Consequently, their ionization may restrict their crossing through the blood-brain barrier (BBB) (Hörter and Dressman, 2001).

In terms of medicinal chemistry, detailed knowledge of the dissociation constants might provide hints helpful to design a new derivative of a drug which could be more effective or less toxic. Regarding aminoglycosides, studying the pK_a values of amines on these alkaloids will afford a better understanding of their structure-activity relationships (SAR), especially the order in which these similar functional groups gain/lose protons. Such data may potentially help in understanding the order of target mRNA binding of key basic functional groups (Hörter and Dressman, 2001; Krężel et al., 2004; Manallack, 2007). Moreover, the specific or selective reactions of different amines located around aminoglycosides, e.g. with FITC, depending on steric effects and the ionisation constants of these amines, may provide an insight into understanding their distribution in the body and mechanisms of toxicity.

1.2.2. Methods for measuring ionisation constants

A number of techniques have been devised for measuring pK_a, including potentiometry, ultraviolet (UV) spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy. All these methods have their own principles, uses and limitations. Their principles, advantages and

limitations are introduced below (Albert and Serjeant, 1984; Box et al., 2008; Blagbrough et al., 2011; Reijenga et al., 2013).

1.2.2.1. Potentiometry

Although it is one of the most convenient ways to measure pK_a as well as the required purity of the sample, a large sample size is needed compared to NMR spectroscopy technique. In the beginning, it was difficult to obtain the correct measurement of pH from the sigmoidal curve, but the process has been automated and software has been developed to reduce this particular type of error. Moreover, where a molecule contains multiple functional groups of a similar type, potentiometric titration cannot identify unambiguously the site of protonation. Therefore, it is not recommended to be used for measuring the ionisation constants of functional groups in complex compounds, such as aminoglycosides. Apart from all the difficulties and disadvantages, the simplicity and low cost of the potentiometric titration method of measuring dissociation constants mean that it has remained one of the most common methods for structurally relatively simple substances that are available in a pure form, in large amounts (Box et al., 2008; Reijenga et al., 2013).

1.2.2.2. Ultraviolet-Visible (UV-Vis) spectroscopy

A change in acidity leads to a change of colour in natural substances, which contain a chromophore. The ionisation of the molecule leads to a change in the spectrum of dissociated and non-dissociated molecules. This principle is employed in the UV-Vis spectrometer to measure the pK_a of substances. Initially, visible light was used to measure the pK_a of acid/base indicators; the technique was extended to be applied to other substances by using UV light (Box et al., 2008; Reijenga et al., 2013). The absorption is measured using two wavelengths across a pH range, where the second wavelength is used to provide line of reference. The ratio of absorption at the two wavelengths is plotted against the pH of the solution. The plot is in the form of a sigmoid curve and the pK_a of the functional group can be estimated from the

inflexion point. This method was first developed by Holmes and Snyder, (1925). The method holds the advantage of being fast, has a good accuracy and can be automated to reduce errors. However, the UV spectrophotometric pH titration method cannot provide the site of protonation where the molecule contains several similar functional groups. Therefore, it cannot be used for measuring the ionisation constants of specific functional groups, which contain several such functional groups compounds (Box et al., 2008; Reijenga et al., 2013).

Moreover, this method cannot be used for measuring the ionisation constants of substances lacking visible or UV chromophores, such as aminoglycoside antibiotics. Thus, there are no obvious published data about measuring the ionisation constants of aminoglycoside antibiotics (Blagbrough et al., 2011).

1.2.2.3. Nuclear Magnetic Resonance (NMR) spectroscopy

NMR spectroscopy is another way to measure pK_a. It is based on the principle that some nuclei have a charge and a spin. The NMR chemical shifts of a compound depend upon their magnetic and therefore chemical environment. Consequently, gradual change of the acidity or basicity leads to alterations in their chemical shifts (δ) (see Fig. 1.10). This phenomenon is sometimes used to determine the ionisation constants. These chemical shifts are plotted against the pH; the pK_a values can be extracted from the inflection points of the sigmoidal curves. The technique was first reported by Grunwald to determine the pK_a of amines in 1957 (Grunwald et al., 1957; Frassineti et al., 1995; Fărcaşiu et al., 1996; Gift et al., 2012; Reijenga et al., 2013; Bezençon et al., 2014).

The main advantage of using NMR spectroscopy over potentiometry or the UV spectroscopy method, where there is a suitable chromophore, is that NMR spectroscopy is a powerful technique when it comes to separating and measuring distinct ionisation constants of functional groups within complex compounds, such as aminoglycoside antibiotics, which have more than one ionisable group (Blagbrough et al., 2011; Reijenga et al., 2013). Therefore,

NMR spectroscopy was used in this research project in order to measure the pK_a values of amines in selected aminoglycosides.

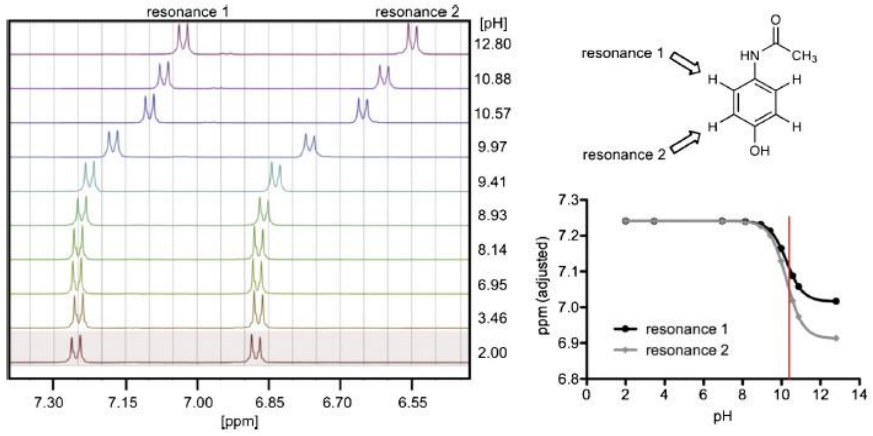


Fig. 1.10 ^{1}H chemical shifts of paracetamol at different pH values in D_2O, the pK_a value of the phenol/phenoxide is extracted from the inflection point of the sigmoidal curves, which is 10.50 (taken from Bezençon et al., 2014).

1.3. Aims and Objectives

The pharmaceutical analysis and medicinal chemistry of a series of aminoglycoside antibiotics consisting of neomycin C (3), paromomycin (4), tobramycin (5), kanamycin B (6), netilmicin (7), sisomicin (8), amikacin (9), and streptomycin (10) will be investigated in this full-time MPhil. In these investigations, we will:

A. Obtain the literature pK_a data of these aminoglycosides and have a focus on the specificity of their assignment, together with the accuracy of their assignments using potentiometry and different NMR nuclei: ^{1}H, ^{13}C, and ^{15}N.

B. Measure the individual pK_a values using combinations of ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopic data, in order to separate the many and varied ionisation constants.

C. Investigate a few specific or selective reactions of different amines located around tobramycin with amino acid protecting groups and with fluorophores. These are preliminary synthetic studies with a view to the synthesis of biologically relevant fluorescently tagged tobramycin.

Chapter 2

Literature Review of the pK_a Values of Amino Groups on Aminoglycoside Antibiotics Measured using Potentiometry and NMR Spectroscopy

Examples of all published measurements of the pK_a values of the amino groups on 2-deoxystreptamine (1), neamine (2), neomycin (3), paromomycin (4), tobramycin (5), kanamycin A and B (6), sisomicin (8), amikacin (9), streptomycin (10), gentamicin C1, C1a, and C2 (11) using potentiometry and NMR spectroscopy are presented in sections 2.1 to 2.10.

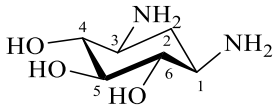
In the literature, all potentiometric titrations were performed using a pH meter fitted with a glass electrode and another electrode as an indicator. The majority of the measurements were fully automated under the control of different kinds of software, such as Matlab 6.5.1 or HYPERQUAD. All potentiometric experiments were carried out at 25°C (unless otherwise indicated) in aqueous solutions at different concentrations as indicated some equivalents of HCl were added to produce a completely protonated form of the antibiotic at the start of titration. Then, the solution was titrated with aqueous NaOH using an autotitrator.

NMR spectroscopy is another way to measure pK_a values. Generally, the ionisation constants were measured using variations in the chemical shift (δ) with ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopies. The chemical shifts were then plotted against the pH, and the pK_a values are extracted from the inflection points of the sigmoidal curves. In the literature, the pK_a values of some aminoglycosides, neomycin (3), paromomycin (4), tobramycin (5), amikacin (9), gentamicin C1, C1a, and C2 (11), were measured using 1D ^{15}N NMR spectroscopy. The 1D ^{15}N measurements were carried out using 15 mm sample tubes that contained concentrated solutions of the aminoglycosides, using uniformly enriched ^{15}N-aminoglycosides, which was prepared from cultures of *Streptomyces* or *Micromonospora* grown in ^{15}N-enriched media, or by increasing the length of time of the acquisition i.e. increasing number of scans. All NMR measurements were carried out referenced relative to HDO, DSS, or TSP signals for ^{1}H and ^{13}C NMR spectroscopy and $^{15}NH_4Cl$, NH_3, or $NH_4{}^{15}NO_3$ for ^{15}N NMR spectroscopy in either

D_2O or different ratios of H_2O/D_2O at 25°C (unless otherwise indicated). The main advantage of using NMR spectroscopy is that it is a powerful technique when it comes to separating and measuring distinct ionisation constants of functional groups within aminoglycosides.

There are no published data about measuring the ionisation constants of aminoglycoside antibiotics using UV-Vis spectrophotometry, because the analytes lack visible or even UV chromophores in their structures. However, there is one paper published by Fuentes et al., (2014) about measuring the pK_a value of N-2' on different hydrazine derivatives of the aldehyde functional groups of streptomycin using UV-Vis spectrophotometry (see section 2.10).

2.1. 2-Deoxystreptamine (1)

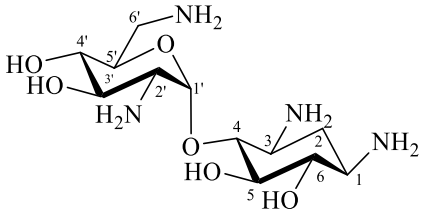


Individual pK_a values / Method	pK_a 1	pK_a 2
Potentiometry [a]	8.95	7.14
Potentiometry [b]	9.12	7.45

[a] pK_a values of amines of 0.01 M 2-deoxystreptamine (1) determined using potentiometry in H_2O at 25°C (Inouye, 1968)

[b] pK_a values of amines of 0.10 M 2-deoxystreptamine (1) determined using potentiometry in H_2O at 25°C (Baran et al., 2001)

2.2. Neamine (2)



Individual pK_a values / Method	pK_a 1	pK_a 2	pK_a 3	pK_a 4
Potentiometry [a]	not reported	8.62	7.73	6.35
Potentiometry [b]	9.43	8.62	7.73	6.35
Potentiometry [c]	9.23	8.31	6.98	6.00

[a] pK_a values of amines of 0.001 M neamine (2) determined using potentiometry in H_2O at 25°C (Sutrisno et al., 2001)

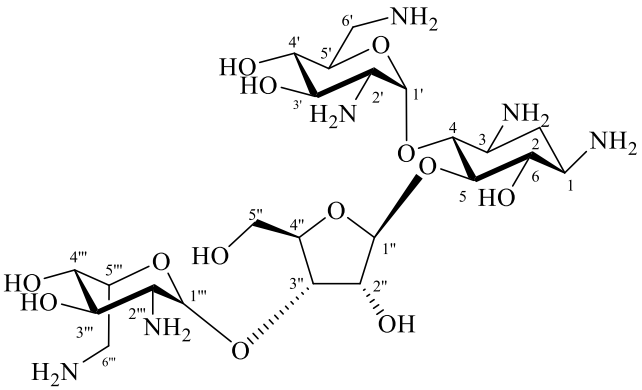
[b] pK_a values of amines of 0.10 M neamine (2) determined using potentiometry in H_2O at 25°C (Baran et al., 2001)

[c] pK_a values of amines of 0.10 M neamine (2) determined using potentiometry in H_2O at 25°C (Tiwow, 2014)

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'	N-6'
^{1}H NMR [a]	7.77	6.44	7.23	8.08

[a] pK_a values of individual nitrogen atoms of 0.01 M neamine (2) determined using ^{1}H NMR spectroscopy in D_2O relative to the HDO peak at 25°C (Andac et al., 2011)

2.3. Neomycin (3)



Individual pK_a values / Method	pK_a 1	pK_a 2	pK_a 3	pK_a 4	pK_a 5	pK_a 6
Potentiometry [a]	9.72	9.24	8.61	8.05	7.55	6.30
Potentiometry [b]	9.37	8.74	8.14	7.60	7.20	5.69
Potentiometry [c]	8.78	8.43	8.18	7.64	7.05	5.69

[a] pK_a values of amines of 0.01 M neomycin (3) determined using potentiometry in H_2O at 25°C (Baran et al., 2001)

[b] pK_a values of amines of 0.15 M neomycin (3) determined using potentiometry in H_2O at 23°C (Jeżowska et al., 2005)

[c] pK_a values of amines of 0.15 M neomycin (3) determined using potentiometry in H_2O at 25°C (Avdeef, 2012)

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'	N-6'	N-2'''	N-6'''
^{15}N NMR [a]	8.04	5.74	7.55	8.60	7.60	8.80
^{15}N NMR [b]	8.70	6.90	8.30	9.20	8.30	9.50
^{15}N NMR [c]	7.90	5.70	7.40	8.10	7.70	8.70
^{1}H NMR [d]	8.10	5.40	7.60	8.70	7.50	8.80

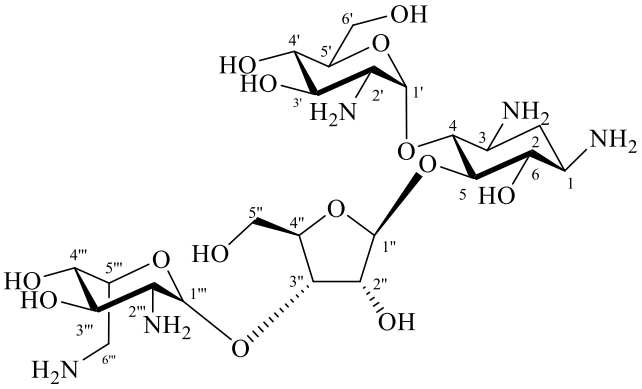
[a] pK_a values of individual nitrogen atoms of 0.10 M neomycin (3) determined using ^{15}N NMR spectroscopy in H_2O/D_2O (85: 15 v/v) relative to $NH_4{}^{15}NO_3$ at 25°C (Botto and Coxon, 1982)

[b] pK_a values of individual nitrogen atoms of 0.45 M neomycin (3) determined using ^{15}N NMR spectroscopy in H_2O/D_2O (90:10 v/v) relative to NH_3 by using 1.0 M ^{15}N urea in DMSO at 25°C (Kaul et al., 2003)

[c] pK_a values of individual nitrogen atoms of 0.001 M ^{15}N-neomycin (3) determined using ^{15}N NMR spectroscopy in H_2O/D_2O (90: 10 v/v) relative to $^{15}NH_4Cl$ at 25°C (Özen et al., 2006)

[d] pK_a values of individual nitrogen atoms of 0.01 M neomycin (3) determined using ^{1}H-^{13}C HSQC NMR spectroscopy in D_2O relative to TSP at 25°C (Freire et al., 2007)

2.4. Paromomycin (4)



Individual pK_a values / Method	pK_a 1	pK_a 2	pK_a 3	pK_a 4	pK_a 5
Potentiometry [a]	8.90	8.23	7.57	7.05	5.99

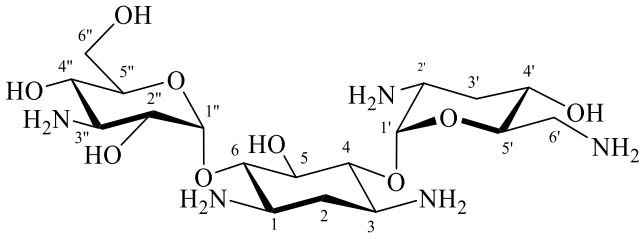
[a] pK_a values of amines of 0.15 M paromomycin (4) determined using potentiometry in H_2O at 37°C (Avdeef, 2012)

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'	N-2'''	N-6'''
^{15}N NMR [a]	8.65	7.07	8.33	8.25	9.46
^{15}N NMR [b]	8.20	6.50	8.07	7.91	9.13

[a] pK_a values of individual nitrogen atoms of 0.45 M paromomycin (4) determined using ^{15}N NMR spectroscopy in H_2O/D_2O (85: 15 v/v) relative to NH_3 using 1 M [^{15}N] urea in DMSO at 25°C (Kaul et al., 2003)

[b] pK_a values of individual nitrogen atoms of 0.01 M paromomycin (4) determined using ^{15}N NMR spectroscopy in H_2O/D_2O (85: 15 v/v) relative to NH_3 using 1 M [^{15}N] urea in DMSO at 25°C (Barbieri and Pilch, 2006)

2.5. Tobramycin (5)



Individual pK_a values / Method	pK_a 1	pK_a 2	pK_a 3	pK_a 4	pK_a 5
Potentiometry [a]	9.00	8.38	7.71	7.10	6.10

[a] pK_a values of amines of 0.10 M tobramycin (5) determined using potentiometry in H_2O at 25°C (Jeżowska et al., 1998)

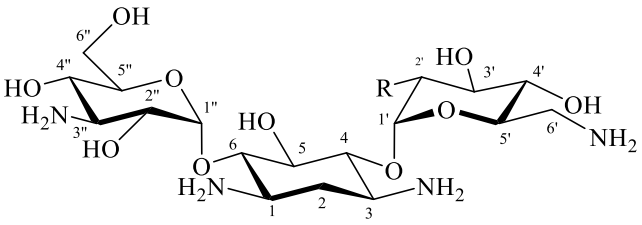
Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'	N-6'	N-3''
^{15}N NMR [a]	7.40	6.20	7.60	8.60	7.40
^{1}H NMR [b]	7.30	6.60	7.50	8.40	7.30
^{15}N NMR [c]	7.40	6.40	7.70	8.50	7.40

[a] pK_a values of individual nitrogen atoms of 0.15 M tobramycin (5) determined using ^{15}N NMR spectroscopy in H_2O/D_2O (90: 10 v/v) relative to $^{15}NH_4Cl$ at 25°C (Dorman et al., 1976)

[b] pK_a values of individual nitrogen atoms of 0.10 M tobramycin (5) determined using ^{1}H NMR spectroscopy in D_2O relative to TMS at 25°C (Pagano et al., 2011)

[c] pK_a values of individual nitrogen atoms of 0.80 M tobramycin (5) determined using ^{15}N NMR spectroscopy in H_2O/D_2O (90: 10 v/v) relative to $^{15}NH_4Cl$ at 25°C (Pagano et al., 2011)

2.6. Kanamycin A and B (6)



Kanamycin A: R = OH
B: R = NH_2

Individual pK_a values / Method	pK_a 1	pK_a 2	pK_a 3	pK_a 4	pK_a 5
Potentiometry [a]	9.12	8.25	7.60	6.91	5.74
Potentiometry [b]	9.03	8.16	7.42	6.19	-
Potentiometry [c]	9.16	8.27	7.52	6.28	-

[a] pK_a values of amines of 0.10 M kanamycin B (6) determined using potentiometry in H_2O at 25°C (Jeżowska et al., 1998)

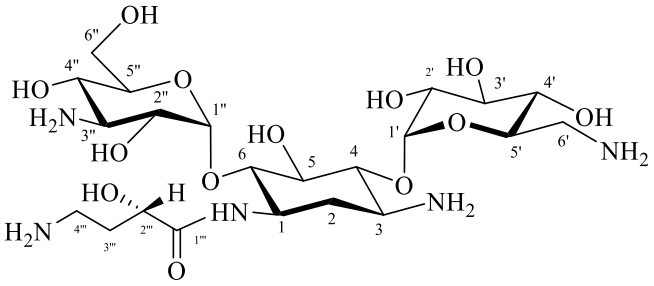
[b] pK_a values of amines of 0.001 M kanamycin A (6) determined using potentiometry in H_2O at 25°C (Szczepanik et al., 2002)

[c] pK_a values of amines of 0.003 M kanamycin A (6) determined using potentiometry in H_2O at 23°C (Fuentes et al., 2010a)

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-6'	N-3''
^{1}H NMR [a]	8.12	6.04	9.03	7.46

[a] pK_a values of individual nitrogen atoms of 0.01 M kanamycin A (6) determined using ^{1}H NMR spectroscopy in 99.9% D_2O relative to TSP at 25°C (Gutiérrez-Moreno et al., 2012)

2.7. Amikacin (9)



Individual pK_a values / Method	pK_a 1	pK_a 2	pK_a 3	pK_a 4
Potentiometry [a]	9.90	8.89	7.81	6.83
Potentiometry [b]	9.78	8.91	7.78	6.88
Potentiometry [c]	9.49	8.77	7.84	7.34

[a] pK_a values of amines of 0.10 M amikacin (9) determined using potentiometry in H_2O at 25°C (Jeżowska and Bal, 1998)

[b] pK_a values of amines of 0.06 M amikacin (9) determined using potentiometry in H_2O at 25°C (Fuentes et al., 2010b)

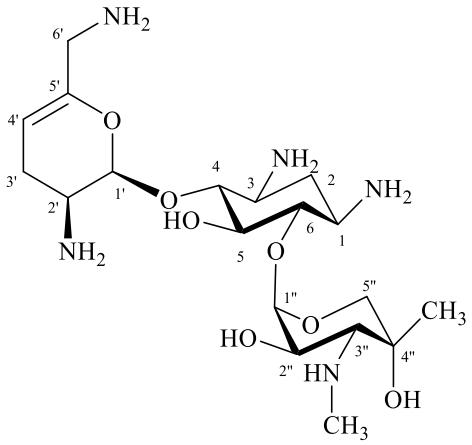
[c] pK_a values of amines of 0.001 M amikacin (9) determined using potentiometry in H_2O at 25°C (Alekseev and Markova, 2016)

Individual nitrogen atoms pK_a / Method	N-3	N-6'	N-3''	N-4'''
^{13}C NMR [a]	7.40	9.50	8.50	10.10
^{15}N NMR [b]	7.62	8.92	8.13	9.70

[a] pK_a values of individual nitrogen atoms of 0.1 M amikacin (9) determined using ^{13}C NMR spectroscopy in H_2O relative to TSP at 25°C (Gaggelli et al., 1995)

[b] pK_a values of individual nitrogen atoms of 0.8 M amikacin (9) determined using ^{15}N NMR spectroscopy in H_2O/D_2O (85: 15 v/v) relative to $^{15}NH_4Cl$ at 27°C (Cox and Serpersu, 1997)

2.8. Sisomicin (8)



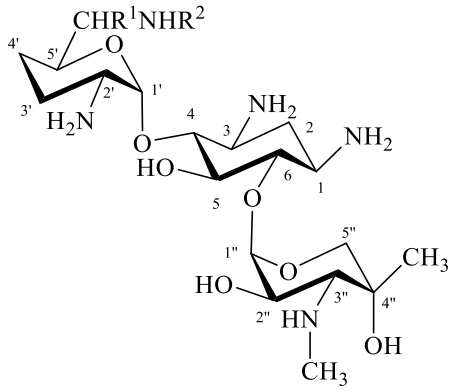
Individual pK_a values / Method	pK_a 1	pK_a 2	pK_a 3	pK_a 4	pK_a 5
Potentiometry [a]	9.41	8.65	7.98	7.25	6.08

[a] pK_a values of amines of 0.10 M sisomicin (8) determined using potentiometry in H_2O at 25°C (Krężel et al., 2004)

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'	N-6'	N-3''
^{1}H NMR [a]	7.34	6.11	7.93	9.45	8.63

[a] pK_a values values of individual nitrogen atoms of 0.01 M sisomicin (8) determined using ^{1}H NMR spectroscopy in D_2O relative to TSP at 25°C (Krężel et al., 2004)

2.9. Gentamicin C1, C1a, and C2 (11)



Gentamicin C1: $R^1 = R^2 = CH_3$
C1a: $R^1 = R^2 = H$
C2: $R^1 = CH_3 = R^2 = H$

Individual pK_a values / Method	pK_a 1	pK_a 2	pK_a 3	pK_a 4	pK_a 5
Potentiometry [a]	9.86	8.81	8.12	7.31	5.68
Potentiometry [b]	9.49	8.86	8.18	7.38	5.76
Potentiometry [c]	9.59	8.79	8.21	7.42	5.83

[a] pK_a values of amines of 0.01 M gentamicin C1 (11) determined using potentiometry in H_2O at 25°C (Lesniak et al., 2003)

[b] pK_a values of amines of 0.01 M gentamicin C1a (11) determined using potentiometry in H_2O at 25°C (Lesniak et al., 2003)

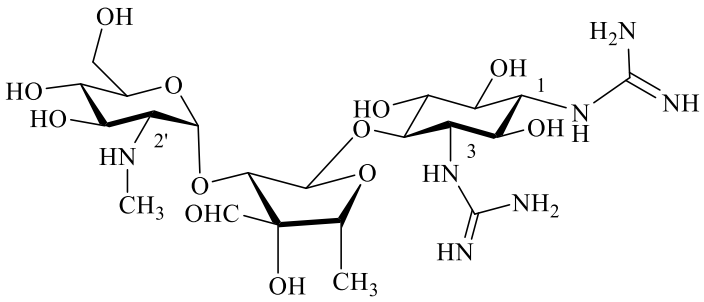
[c] pK_a values of amines of 0.01 M gentamicin C2 (11) determined using potentiometry in H_2O at 25°C (Lesniak et al., 2003)

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'	N-6'	N-3''
^{1}H NMR [a]	7.67	6.19	7.40	9.86	8.78
^{15}N NMR [b]	8.91	7.22	8.17	9.99	9.25

[a] pK_a values of individual nitrogen atoms of 0.001 M gentamicin C-1 determined using ^{1}H NMR spectroscopy in 99.9% D_2O relative to TSP at 25°C (Lesniak et al., 2003)

[b] pK_a values of individual nitrogen atoms of 0.8 M gentamicin C (mixture) determined using ^{15}N NMR spectroscopy in H_2O/D_2O (85: 15 v/v) relative to $^{15}NH_4Cl$ at 25°C (Lesniak et al., 2003)

2.10. Streptomycin (10)



Individual pK_a values / Method	pK_a 1	pK_a 2	pK_a 3
Potentiometry [a]	7.66	not reported	not reported
Potentiometry [b]	7.94	not reported	not reported
Potentiometry [c]	7.91	not reported	not reported

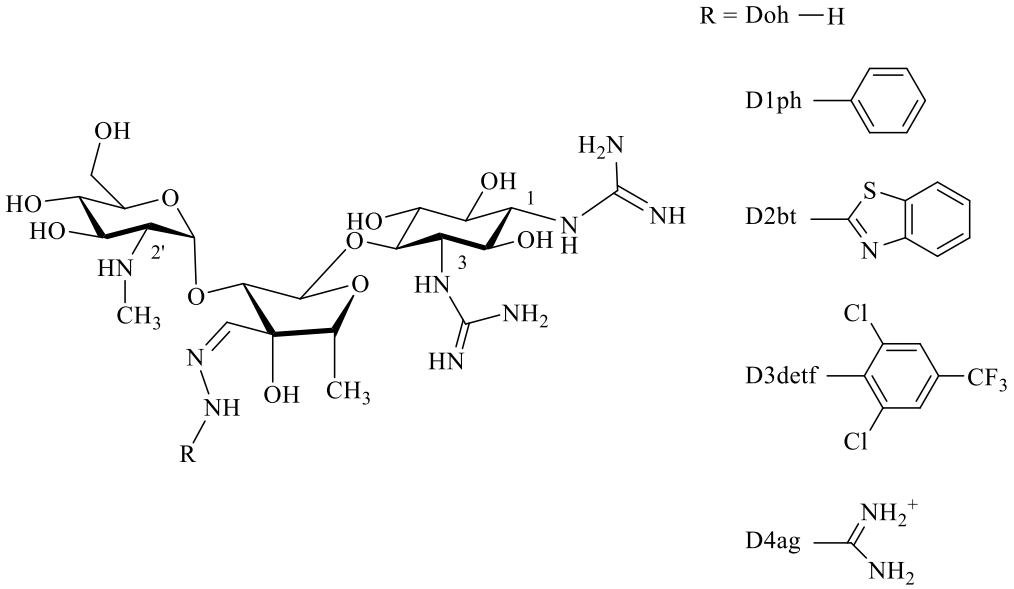
[a] pK_a values of amines of streptomycin (10) HCl determined using potentiometry (Kuehl et al., 1946)

[b] pK_a values of amines of 0.02 M streptomycin (10) determined using potentiometry in H_2O at 25°C (Fuentes et al., 2010b)

[c] pK_a values of amines of 0.008 M streptomycin (10) determined using potentiometry in H_2O at 25°C (Fuentes et al., 2014)

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'
^{1}H NMR [a]	13.55	12.33	8.29

[a] pK_a values of individual nitrogen atoms of 0.001 M streptomycin (10) determined using ^{1}H NMR spectroscopy in H_2O/D_2O (95: 05 v/v) relative to DSS at 25°C (Orgován and Noszál, 2012)



Individual pK_a values / Method	pK_a 1	pK_a 2	pK_a 3
UV-Vis [a]	7.48	not reported	not reported

[a] pK_a values of amines of hydrazine derivatives of streptomycin (10) determined using UV-Vis spectrophotometry (Fuentes et al., 2014)

In conclusion, although potentiometry is one of the most convenient and automated methods to measure pK_a values, potentiometric titration cannot identify unambiguously the site of protonation. It can be easily seen that the pK_a values of some amines on some aminoglycoside antibiotics obtained using potentiometry are inconsistent. For example, the pK_a values of six amines of neomycin reported by Baran et al., (2001) are 9.37, 8.74, 8.14, 7.60, 7.20, and 5.69, However, the pK_a values of the same amines on the same compound of neomycin reported by Avdeef et al., (2012) are 8.78, 8.43, 8.18, 7.64, 7.05, and 5.69 in the

same solvent (H_2O), at 25°C. The difference in the concentration (0.01 M of neomycin reported by Baran et al., (2001) and 0.15 M of neomycin reported by Avdeef et al., (2012)) could explain the difrences in the pK_a values reported. The variation observed in the pK_a values could be due to the differences in the concentrations, temperatures, and NMR instruments. When the literature on the determination of pK_a values of amines on aminoglycoside antibiotics is examined, it can be seen that there are some amines on some aminoglycosides not reported, such as an amine on neamine and the two guanidine groups on streptomycin.

The main advantage of using NMR spectroscopy over potentiometry or the UV spectroscopy method is that NMR spectroscopy can identify unambiguously the site of protonation within complex compounds, such as aminoglycoside antibiotics. In the literature, the amines on some aminoglycosides, such as neomycin (3), paromomycin (4), tobramycin (5), amikacin (9), gentamicin C1, C1a, and C2 (11), were measured using 1D ^{15}N NMR spectroscopy. However, it is not impossible but it is difficult to assign the individual nitrogen atoms on aminoglycosides using 1D ^{15}N NMR spectroscopy. The assignments of individual nitrogen atoms on aminoglycosides using 1D ^{15}N NMR spectroscopy have been discussed (Dorman et al., 1976; Botto and Coxon, 1982). There is only one published article (Gaggelli et al., 1995) for measuring the pK_a values of amines on an aminoglycoside using ^{13}C NMR spectroscopy. Those studies were on amikacin (9).

In this thesis, the pK_a values of 2-deoxystreptamine (1), neamine (2), neomycin C (3), paromomycin (4), tobramycin (5), kanamycin B (6), netilmicin (7), sisomicin (8), amikacin (9), and streptomycin (10) were measured using new combinations of ^{1}H, ^{13}C, and ^{15}N HMBC NMR data, in order to separate the many and varied ionisation constants. Measuring the pK_a values using ^{15}N HMBC NMR spectroscopy has not been previously reported. The advantages of using ^{15}N HMBC over 1D ^{15}N NMR spectroscopy are that the assignment of individual nitrogen atoms on aminoglycosides using ^{15}N HMBC spectroscopy is much easier than using 1D ^{15}N NMR spectroscopy. Moreover, the length of time required for the acquisition of ^{15}N HMBC data points (55 min) is significantly less than that required for 1D ^{15}N NMR spectroscopy (2h).

Chapter 3

Experimental

3.1. Materials and General Methods

Deuterium oxide (D_2O), CD_3OD, $CDCl_3$, DCl and NaOD were purchased from Goss Scientific. The purchased DCl was a 20% concentration solution in D_2O. NaOD was a 30% concentration solution in D_2O. Anhydrous methanol, ethyl acetate, dichloromethane (DCM), triethylamine (Et_3N), dimethylformamide (DMF) and aqueous ammonia (32%) were purchased from VWR. Neamin free base (2) was purchased from Carbosynth. 2-Deoxystrptamine (1), neomycin sulfate (3), paromomycin sulfate (4), tobramycin sulfate (5), kanamycin B sulfate (6), netilmicin sulfate (7), sisomicin sulfate (8), amikacin sulfate (9), streptomycin sulfate (10), potassium hydrogen phthalate, di-sodium tetra-borate, fluorescein isothiocyanate (FITC), ninhydrin, di-*tert*-butyl dicarbonate ($(Boc)_2O$), *N*-(benzyloxycarbonyloxy)-phthalimide(*O*-Cbz-*N*-hydroxyphthalimide) trimethylsilylpropanoic acid (TMSP), nitromethane (CH_3NO_2), sodium sulfate (Na_2SO_4), and potassium carbonate (K_2CO_3) were purchased from Sigma-Aldrich.

Column chromatography has been performed over silica gel 60-120 mesh (purchased from Sigma-Aldrich) using different ratios of methanol, ethyl acetate, dichloromethane (DCM), aqueous ammonia (32%), and triethylamine (Et_3N) as eluents.

Thin-Layer Chromatography (TLC) over silica gel has been performed using aluminium-backed sheets coated with Kieselgel 60 F_{254} purchased from Merck. Ninhydrin TLC spray reagent was used for detecting amine functional groups (ninhydrin (0.2 g) in 100 mL ethanol).

3.2. Instrumentation

NMR spectra including ^{1}H, ^{13}C, HSQC, HMBC, NOESY, and ^{15}N HMBC were recorded on Bruker Avance III (operating at 500.13 MHz for ^{1}H, 125.77 MHz for ^{13}C, and 50.67 MHz for ^{15}N HMBC) spectrometers at 25°C (unless otherwise indicated). MestReNova and Bruker Topspin have been used for processing the spectra. ^{1}H and ^{13}C chemical shifts (δ) were observed and are reported in parts per million (ppm) relative to trimethylsilylpropanoic acid (TMSP) at 0.00 ppm as an internal reference and ^{15}N HMBC chemical shifts were measured relative to external nitromethane (CH_3NO_2 in $CDCl_3$ (1:1, v/v) at -511.72 ppm (Wishart et al., 1995). The total recording time differs for each isotope as follows: less than 2 min, 30 min, and 60 min per data point for ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy, respectively.

High Resolution Time-of Flight (HR TOF) mass spectra were obtained on a Bruker Daltonics "micrOTOF" mass spectrometer using electrospray ionisation (ESI) (loop injection +ve mode).

3.3. Calibration of 5mm NMR tube-pH electrode

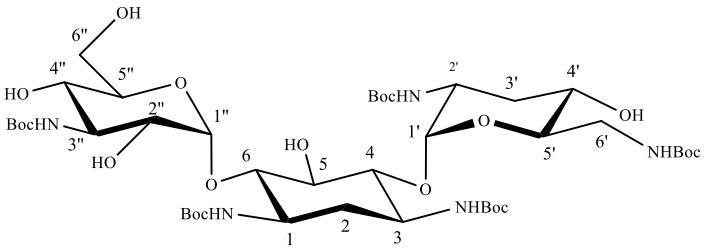
A 5mm NMR tube-pH electrode purchased from Sigma-Aldrich, was used for measuring pH values. The electrode easily fitted into the 5mm NMR tube. Standard buffers consisting of 0.40 M potassium hydrogen phthalate in H_2O, pH 4.00 and 0.01 M di-sodium tetra-borate in H_2O, pH 9.18 were used for calibrating the 5mm NMR tube-pH electrode. All the measurements were carried out at 25°C.

3.4. pK_a determination using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy

Aminoglycoside analyte solutions were prepared at ~10 mg/mL concentration. These solutions typically started at ~0.7 M analyte, beginning from an acidic pH and adjusting with 0.5 M NaOD to pH = 14, when the final concentration will have been diluted to ~0.2 M.

Experiments were within the range 1.00-0.15 M aminoglycoside in 99.97% D_2O and 100% H_2O. The pH values were adjusted using 0.5 M NaOD and 0.5 M DCl (for measuring pK_a in 99.97% in D_2O) and 0.5 M NaOH and 0.5 M HCl (for measuring pK_a in 100% in H_2O). MestReNova and Bruker Topspin were used for analysis of the recorded spectra. Chemical shifts of ^{1}H, ^{13}C, and ^{15}N HMBC of aminoglycosides at varying pH values were plotted against pH values. The nonlinear sigmoidal curve and the inflection point of the sigmoidal curve were determined using GraphPad Prism 7 (Version 2017), after subtraction of 0.5 to convert the measured pD values into pH values (Popov et al., 2006). The pK_a values of individual nitrogen atoms of aminoglycosides are extracted from the inflection points of the sigmoidal curves.

3.5. Synthesis of compound 12



12

This synthesis was carried out following the protocols in Michael et al. (1999) with modification such that tobramycin was used instead of neomycin and 5.0 equiv. was used instead of 6 equiv. A solution of tobramycin (5) (0.405 g, 0.866 mmol) in a mixture of DMF, water, and triethylamine (2: 1: 0.02 v/v/v) was treated with di-*tert*-butyldicarbonate $(Boc)_2O$ (0.945 g, 4.331 mmol, 5 equiv.). The reaction was maintained at 60°C for 24h. The volatiles were removed in vacuo. The residue was partitioned between ethyl acetate and water (2:1 v/v). The aqueous layer was extracted with ethyl acetate (7 x 150 mL), dried (Na_2SO_4), and then concentrated under reduced pressure. The crude material was subject to column chromatography using dichloromethane (DCM) in methanol (95: 05 v/v). The desired product was obtained as a white solid (0.675 g, 80%). TLC analysis showed one spot (R_f = 0.5, ethyl acetate: aqueous ammonia, 95: 05 v/v).

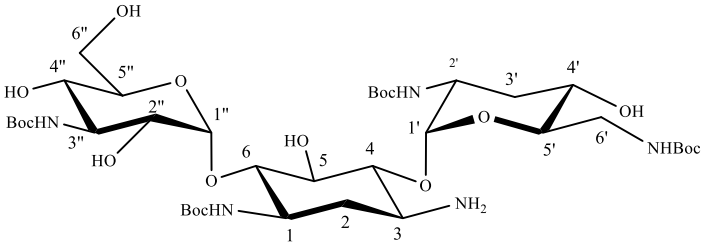
HRMS: Found 990.5203, $C_{43}H_{77}NaN_5O_{19}$ requires 990.5102 $[M + Na]^+$;

IR: 2998 (NH) and 1688 (C=O) cm-1;

^{1}H NMR (500.13 MHz, CD_3OD and $CDCl_3$ (1: 1 v/v)): 1.36-1.50 (overlapping s, 45H, 15 x $C\underline{H}_3$, Boc), 1.49-1.61 (m, 1H, H-2ax), 1.62-1.69 (m, 1H, H-3'ax), 2.04-2.16 (m, 1H, H-3'eq), 2.20-2.29 (m, 1H, H-2eq), 3.25-3.55 (m, 9H, H-1, H-4, H-6, H-2', H-4', H-6'a, H-6'b, H-4", and H-5"), 3.68-3.86 (m, 6H, H-3, H-5, H-2", H-3", H-6"a, and H-6"b), 3.88-3.95 (m, 1H, H-5'), 4.85-5.09 (m, 2H, H-1' and H-1");

^{13}C NMR (125.77 MHz, CD_3OD and $CDCl_3$ (1: 1 v/v)): 30. 29 (15 x $\underline{C}H_3$, Boc) 35.06 (CH_2-3'), 35.98 (CH_2-2), 43.32 (CH_2-6'), 52.52 (CH-3), 52.60 (CH-1 and 2'), 58.24 (CH-3"), 63.68 (CH_2-6"), 67.59 (CH-4'), 71.34 (CH-4"), 73.57 (CH-5"), 75.16 (CH-2"), 76.14 (CH-5'), 78.23 (CH-5), 81.80 (5 x Cq, Boc) 84.57 (CH-4), 85.84 (CH-6), 101.31 (CH-1' and 1"), 160.64 (5 x C=O, Boc).

3.6. Synthesis of compound 13



13

This synthesis was carried out following the protocols in Michael et al., (1999) with modification such that tobramycin was used instead of neomycin, 4.0 equiv. was used instead 6 equiv., and 0℃ was used instead of 60℃. A solution of tobramycin (5) (0.403 g, 0.862 mmol) in a mixture of DMF, water, and triethylamine (2: 1: 0.02 v/v/v) was treated with di-*tert*-butyldicarbonate $(Boc)_2O$ (0.752 g, 3.447 mmol, 4.0 equiv.). The reaction was held at 0°C for 2h and then allowed to warm to 20 °C for a further 22h. The volatiles were removed in vacuo. The residue was partitioned between ethyl acetate and water (2:1 v/v). The aqueous layer was extracted with ethyl acetate (7 x 150 mL), dried (Na_2SO_4), and then concentrated

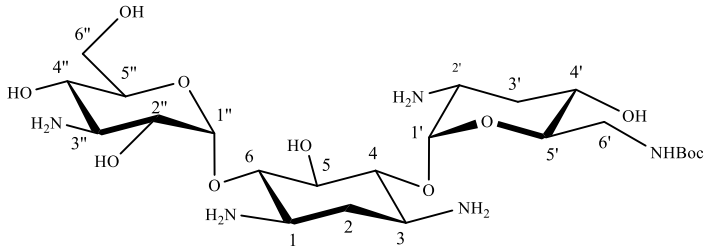
under reduced pressure. The crude material was subject to column chromatography using dichloromethane in methanol (90: 10 v/v). The desired product was obtained as a white solid (0.230 g, 31%). TLC analysis showed one spot (R_f = 0.4, ethyl acetate: ethanol: aqueous ammonia, 7: 2: 1 v/v/v).

HRMS: Found 868.491, $C_{38}H_{70}N_5O_{17}$ requires 868.471 $[M + H]^+$;

^{1}H NMR (500.13 MHz, CD_3OD): 1.26-1.39 (m, 1H, H-2ax), 1.38-1.49 (overlapping s, 36H, 12 x C<u>H</u>$_3$, Boc), 1.60-1.67 (m, 1H, H-3'ax), 2.02-2.17 (m, 2H, H-2eq and 3'eq), 2.73-62.88 (m, 1H, H-3), 3.12-3.48 (m, 6H, H-4, H-4', H-6'a, H-6'b, H-4", and H-5"), 3.47-3.81 (m, 8H, H-1, H-5, H-6, H-2', H-2", H-3", H-6"a, and H-6"b), 3.88-4.02 (m, 1H, H-5'), 4.93-5.01 (m, 1H, H-1"), and 5.04-5.12 (m, 1H, H-1');

^{13}C NMR (125.77 MHz, CD_3OD): 30. 29 (12 x <u>C</u>H_3, Boc), 34.84 (CH_2-3'), 36.96 (CH_2-2), 42.98 (CH_2-6'), 51.28 (CH-1), 52.12 (CH-3), 52.61 (CH-2'), 57.35 (CH-3"), 62.01 (CH_2-6"), 67.02 (CH-4'), 69.73 (CH-4"), 71.97 (CH-5"), 74.45 (CH-5'), 74.68 (CH-2"), 76.10 (CH-5), 80.41 (4 x Cq, Boc), 84.82 (CH-6), 89.81 (CH-4), 99.50 (CH-1'), 100.97 (CH 1"), 158.08 (4 x C=O, Boc).

3.7. Attempted synthesis of compound 14



14

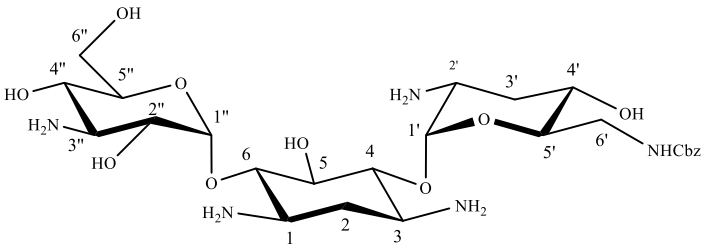
This synthesis was carried out following the protocols in Michael et al., (1999) with modification such that tobramycin was used instead of neomycin, 1.0 equiv. was used instead 6 equiv., and 0°C was used instead of 60°C. A solution of tobramycin (5) (0.502 g, 1.073 mmol) in a mixture of DMF, water, and triethylamine (2: 1: 0.02 v/v/v) was treated with di-*tert*-butyldicarbonate $(Boc)_2O$ (0.234 g, 1.073 mmol, 1.0 equiv.). The reaction was held at 0

°C for 2h and then warmed to 20 °C for a further 22h. TLC analysis showed the presence of several spots and one major spot which was the starting material (methanol: aqueous ammonia 3:2 v/v).

HRMS: showed several peaks (the desired compound 14 was not detected).

^{1}H and ^{13}C NMR spectroscopy showed complicated spectra.

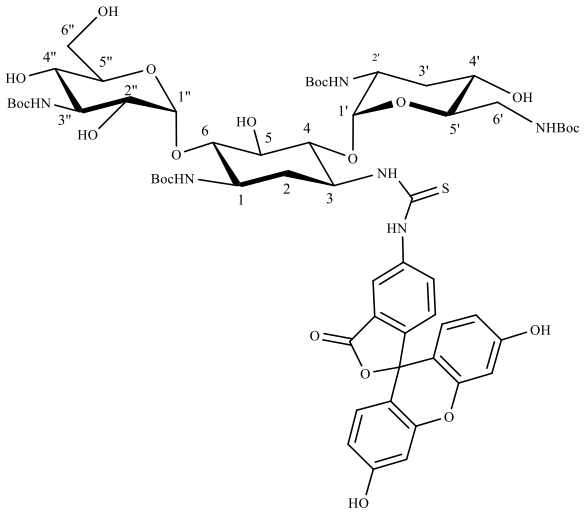
3.8. Attempted synthesis of compound 15



15

This synthesis was carried out following the protocols in Chandrika et al., (2015) except that 0°C was used instead of 20°C. A solution of tobramycin (5) (0.078 g, 0.166 mmol) in a mixture of methanol, water (1:1 v/v), and K_2CO_3 (0.022 g, 0.166 mmol, 1 equiv.) was treated with *N*-(benzyloxycarbonyloxy) phthalimide (*O*-Cbz-*N*-hydroxyphthalimide) (0.049 g, 0.166 mmol, 1.0 equiv.). The reaction was held at 0 °C for 2h and then warmed to 20 °C for a further 22h, and then concentrated under reduced pressure. The desired compound 15 was detected using HRMS: found 624.288, $C_{26}H_{43}NaN_5O_{11}$ requires 624.301 $[M + Na]^+$. However, purification by column chromatography using MeOH and a gradient of triethylamine (0-6%) failed, most likely due to the high polarity of compound 15.

3.9. Attempted synthesis of compound 16



16

This synthesis was carried out following the protocols in Litovchick al., (2001) except that the compound 13 was used instead of neomycin (3). A solution of compound 13 (0.150 g, 0.172 mmol) in a mixture of water/ methanol/ dioxane (1:1:1, v/v/v) was treated with FITC (0.135 g, 0.344 mmol, 2.0 equiv.). The reaction solution was maintained at 60°C for 24h. TLC analysis showed the presence of one major spot, the starting material (ethyl acetate: ethanol: aqueous ammonia, 7: 2: 1 v/v/v).

HRMS: showed the peak of the starting material (compound 13) (the desired compound 16 was not detected).

^{1}H and ^{13}C NMR spectroscopy showed complicated spectra.

Another attempt was carried out following the protocols in Bandyopadhyay et al., (2017) except that the reaction was heated at 80°C for 27h rather than 20°C for 1h. A solution of compound 13 (0.200 g, 0.230 mmol) in a mixture of DMF (2 mL) and Et_3N (1 mL) was treated with FITC (0.179 g, 0.460 mmol, 2.0 equiv.). The reaction solution heated at 80°C for 72h. TLC analysis showed the presence of one major spot, the starting material (compound 13) (ethyl acetate: ethanol: aqueous ammonia, 7: 2: 1 v/v/v).

HRMS: showed the peak of the starting material (compound 13) (the desired compound 16 was not detected). ^{1}H and ^{13}C NMR spectroscopy showed complicated spectra.

Chapter 4

Results and Discussion

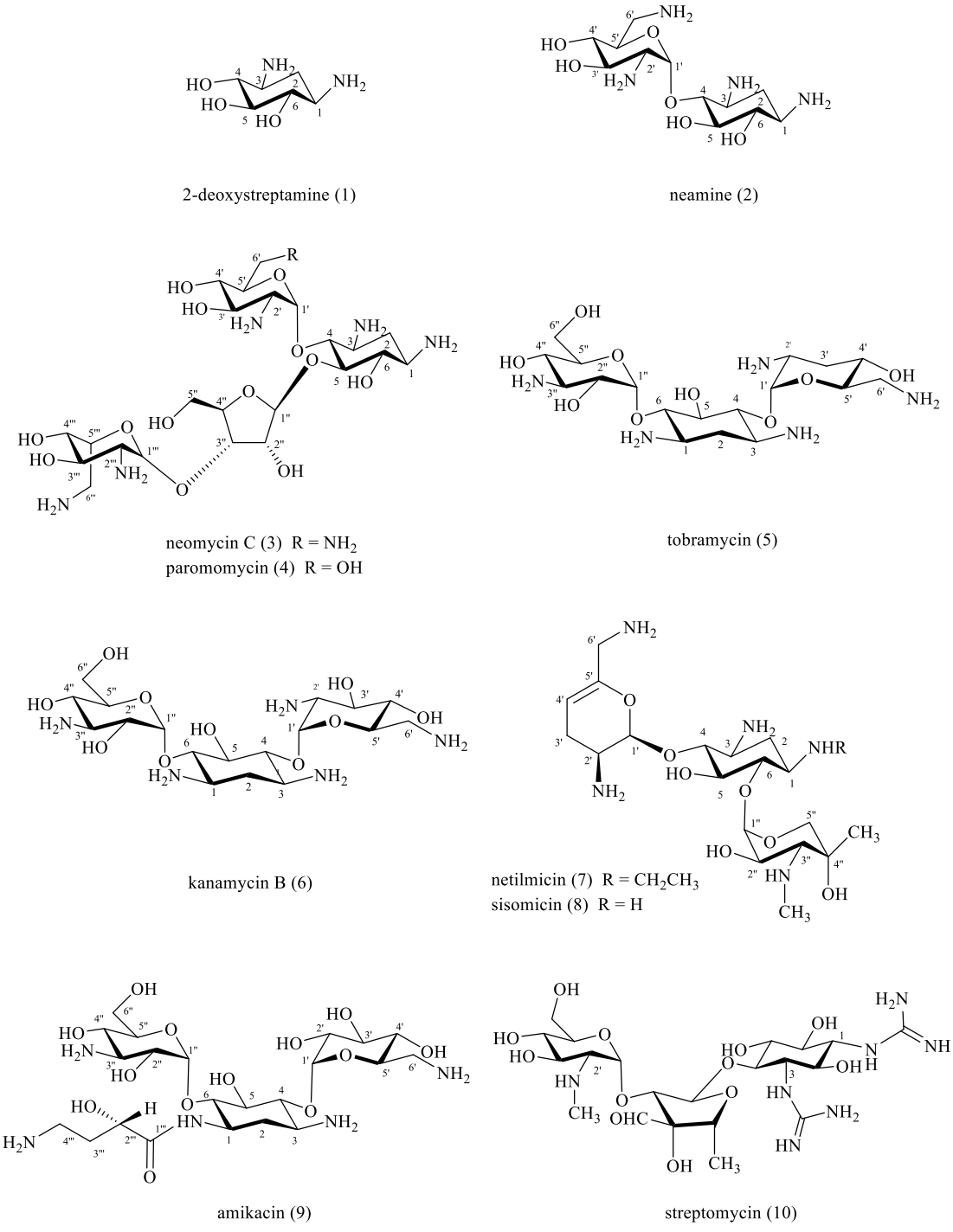


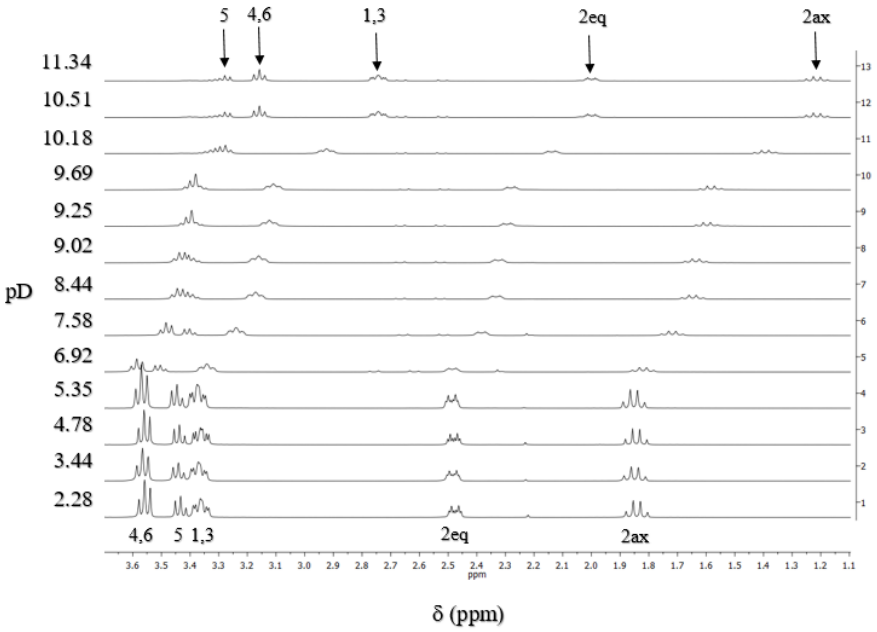
Fig. 4.1 2-Deoxystreptamine (1), neamine (2), neomycin C (3), paromomycin (4), tobramycin (5), kanamycin B (6), netilmicin (7), sisomicin (8), amikacin (9), and streptomycin (10)

The individual ionisation constants, the pK_a values of the individual amino groups of 2-deoxystreptamine (1), neamine (2), neomycin C (3), paromomycin (4), tobramycin (5), kanamycin B (6), netilmicin (7), sisomicin (8), amikacin (9), and streptomycin (10) (see Fig. 4.1) were determined using chemical shift (δ) variation with ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy. The chemical shifts of ^{1}H, ^{13}C, and ^{15}N of these (semi-synthetic and) natural products depend on their chemical environment. Consequently, the gradual change in acidity or basicity leads to subtle alterations in their chemical shifts (δ) (see Fig. 4.2).

The ionisation constants were measured for different kinds of amines on aminoglycoside alkaloids (see Table 4.1). Unambiguous assignments were made for each individual proton, carbon, and amine substituent on these clinically important aminoglycoside antibiotics using ^{1}H, ^{13}C, HSQC, HMBC, NOESY, and ^{15}N HMBC NMR spectroscopy (see Fig. 4.3) . Where proton and carbon signals overlap, ^{1}H-^{13}C HSQC was used to determine the chemical shifts (δ) of each of the protons and the carbons. These chemical shifts were then plotted against the pH; the pK_a values can be extracted from the inflection points of these sigmoidal curves.

The reason for using NMR spectroscopy rather than potentiometry or UV spectrophotometry is that NMR spectroscopy is a powerful technique when it comes to separating and measuring distinct pK_a values of amino groups located around aminoglycoside antibiotics. The NMR signals measured at low pH are diagnostic of the fully (>99%) protonated forms of these amino substituents. On the other hand, the signals obtained at high pH indicate the deprotonated amines on these alkaloids. The ^{1}H and ^{15}N peaks shift down-field with decreasing pH. The ^{1}H and ^{15}N NMR spectroscopic data show that each NH_2 group resonated at lower chemical shifts (δ) (ppm) than its the corresponding protonated amine (NH_3^+); the reverse is true for the ^{13}C chemical shifts (δ).

A



B

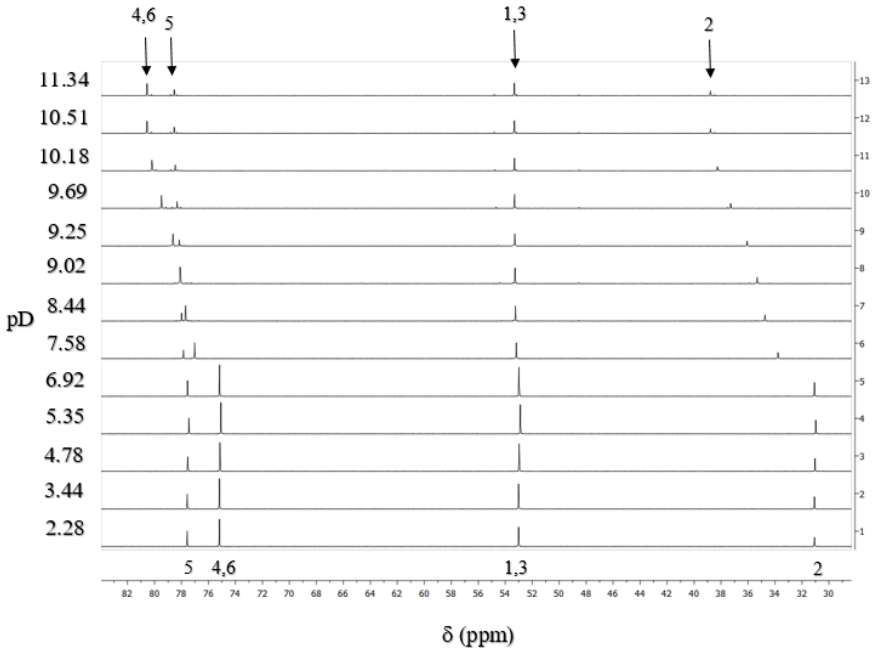


Fig. 4.2 NMR spectra (in D_2O) between pD 2.28 and 11.34 showing chemical shifts δ (ppm) of: A) (^{1}H NMR) H-1/3, H-2ax, H-2eq, H-4/6, H-5 and B) (^{13}C NMR) C-1/3, C-2, C-4/6, and C-5 (marked with arrows) for 2-deoxystreptamine (1)

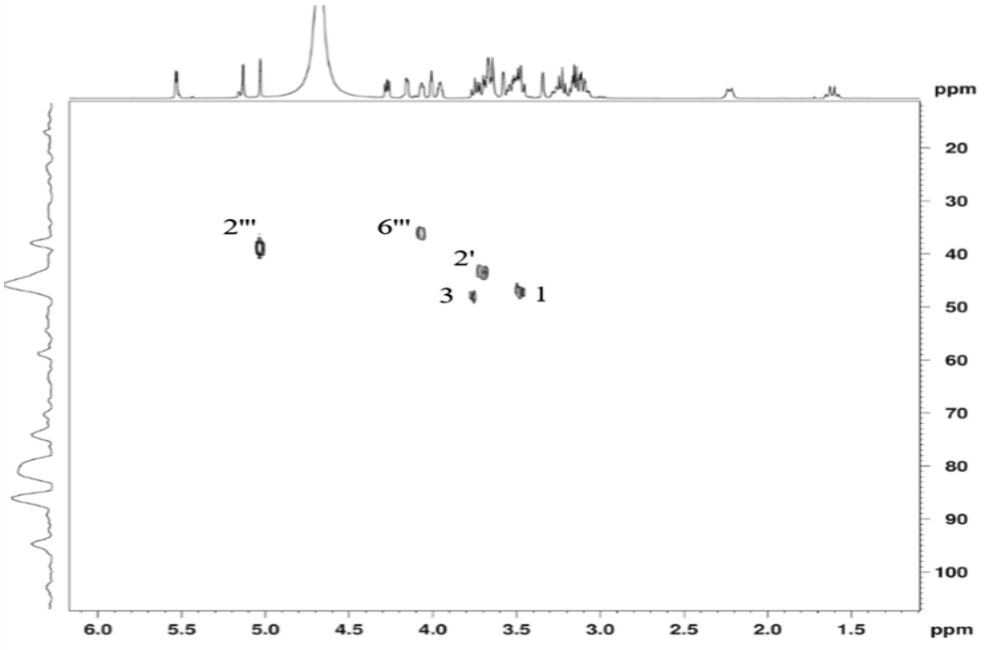


Fig. 4.3 ^{15}**N** HMBC NMR spectrum of paromomycin in D_2O at 25°C at pD 6.50

Table 4.1 The number of amino groups (X) of particular aminoglycoside antibiotics

Aminoglycoside	**X**	**1°**	**2°**	**Side chain**	**Guanidine**	**R-CH_2NH_2**
2-deoxystreptamine	2	2	0	0	0	0
neamine	4	4	0	0	0	1
neomycin C	6	6	0	0	0	2
paromomycin	5	5	0	0	0	1
tobramycin	5	5	0	0	0	1
kanamycin B	5	5	0	0	0	1
amikacin	4	4	0	1	0	0
sisomicin	5	4	1	0	0	1
netilmicin	5	3	2	0	0	1
streptomycin	3	0	1	0	2	0

X. Number of amino groups

1°. Primary amine

2°. Secondary amine

4.1. Determination of the pK_a values of the individual amino groups on neomycin C (3) and tobramycin (5) using ^{1}H NMR spectroscopy in H_2O

D_2O (heavy water) is broadly used in chemistry as a solvent alternative to H_2O. In terms of measuring pK_a values, the comparisons of pH and pD determined data are not straightforward, because the binding affinities of protonating groups are, in general, different for H^+ and D^+. For this reason, the apparent pK_a values, measured in D_2O and expressed using pD, which is a measure of D^+ concentration, are not similar to the corresponding values, measured in H_2O and expressed in pH, which is a measure of H^+ concentration.

The direct determination of pD is not feasible, as pH electrodes are calibrated with buffer solutions in H_2O. Therefore, for heavy water, the actual reading of the pH electrode and pH meter is pD not pH. The relationship between the dissociation of D_2O and H_2O is shown in the following equation:

$$pH = pD - 0.5$$

This equation came about because D_2O is less dissociated than H_2O, as mentioned above (Cook and Lister, 2014). This variation results in 0.50 unit shift between the pH of H_2O and that of the pD for D_2O. According to Popov et al. (2006), there are some factors that affect the correction value (C.V.) (0.5) that need to be subtracted to convert measured pD values to pH, which are the ionic strength of the compound and the temperature of the NMR sample. When these factors increase, the correction value (C.V.) increases.

In order to confirm the validation of the correction value (C.V.), which is 0.5, the pK_a values of the individual amino groups of neomycin C (3) and tobramycin (5) were measured using ^{1}H NMR spectroscopy in 100% H_2O. The pK_a values of amino groups on neomycin C (3) and tobramycin (5) that were measured in 100% H_2O coincide, within the error bars ±0.05, with those determined in 99.97% D_2O (see Fig. 4.4, Tables 4.2, 4.3 for neomycin C and Fig. 4.5, Tables 4.4, 4.5 for tobramycin).

This study confirms that both the use of H_2O solutions for determining pK_a is entirely feasible, and that the use of the correction factor value, although seemingly an arbitrary 0.5

units, is in fact a reasonable approach. Recording NMR spectra in pure protio solvent is not without complications, not least the requirement for solvent suppression, and often NMR spectroscopic radiation damping effects leading to obscured resonances.

Table 4.2 The ^{1}H chemical shifts (ppm) (500 MHz) of H-1, H-3, H-2', H-6'a, H-6'b, H-2''', H-6'''a, and H-6'''b of 0.215-0.120 M neomycin C were measured relative to TMSP in 100% H_2O at 25°C at different pHs

pH	H-1	H-3	H-2'	H-6'a	H-6'b	H-2'''	H-6'''a	H-6'''b
2.00	3.4	3.59	3.46	3.37	3.47	3.61	3.31	3.43
4.98	3.4	3.59	3.46	3.37	3.47	3.61	3.31	3.43
6.71	3.32	3.36	3.42	3.35	3.48	3.55	3.26	3.39
7.58	3.23	3.04	3.25	3.33	3.44	3.45	3.21	3.32
8.55	2.9	2.88	2.91	3.15	3.2	3.21	3.16	3.21
11.01	2.71	2.89	2.74	2.90	3.01	3.03	2.93	3.01

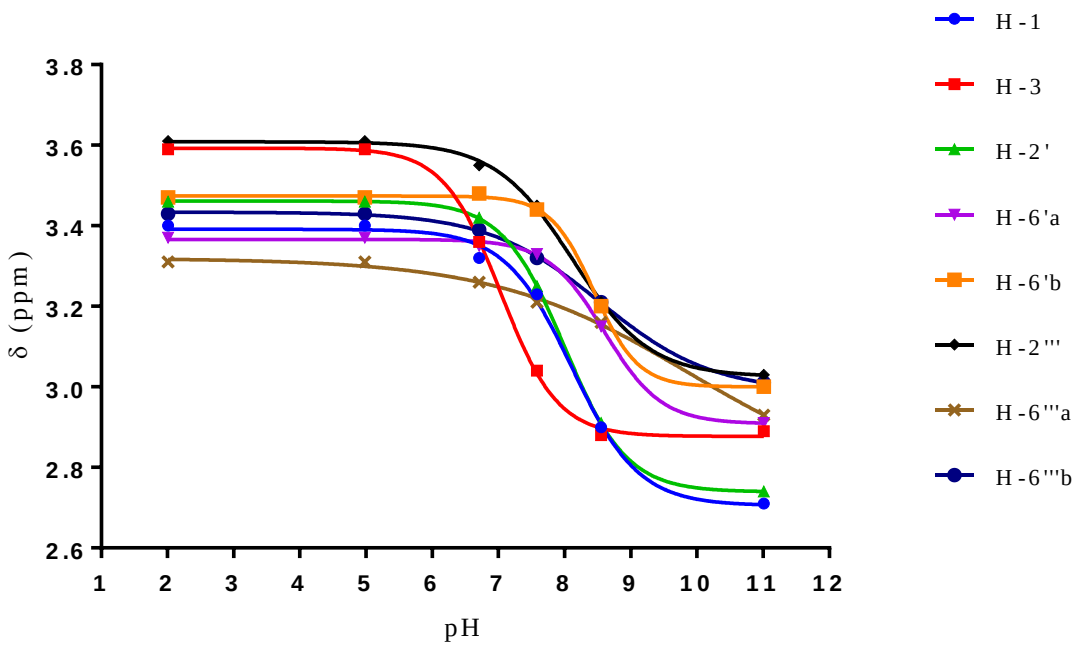


Fig. 4.4 NMR titration curves for the ^{1}H chemical shifts (δ) of 0.215-0.120 M neomycin C (3) were measured relative to TMSP in 100 % H_2O at 25°C

Table 4.3 pK_a values of individual nitrogen atoms of neomycin C in 99.97% D_2O and 100% H_2O, as indicated

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'	N-6'	N-2'''	N-6'''
^{1}H NMR [a]	8.10 ±0.07	6.90 ±0.02	8.00 ±0.05	8.67 ±0.05 [c]	8.05 ±0.05	8.73 ±0.05 [c]
^{1}H NMR [b]	8.05	6.94	8.00	8.62 [d]	8.09	8.67 [d]

[a] This work in 99.97% D_2O

[b] This work in 100% H_2O

[c] The pK_a value of N-6' of neomycin C determined using ^{1}H NMR spectroscopy (in this work in 99.97% D_2O) is the average pK_a of the values of N-6' obtained using ^{1}H NMR spectroscopic data for 6'a (8.65) and 6'b (8.70), and the pK_a value of N-6''' of neomycin C determined using ^{1}H NMR spectroscopy (in this work in 99.97% D_2O) is the average pK_a of the values of N-6''' obtained using ^{1}H NMR spectroscopic data for 6'''a (8.72) and 6'''b (8.75)

[d] The pK_a value of N-6' of neomycin C determined using ^{1}H NMR spectroscopy (in this work in 100% H_2O) is the average pK_a of the values of N-6' obtained using ^{1}H NMR spectroscopic data for 6'a (8.60) and 6'b (8.65), and the pK_a value of N-6''' of neomycin C determined using ^{1}H NMR spectroscopy (in this work in 100% H_2O) is the average pK_a of the values of N-6''' obtained using ^{1}H NMR spectroscopic data for 6'''a (8.65) and 6'''b (8.70)

Table 4.4 The ^{1}H chemical shifts (ppm) (500 MHz) of H-1, H-3, H-2', H-6'a, H-6'b, and H-3'' of 0.250-0.130 M tobramycin were measured relative to TMSP in 100 % H_2O at 25°C at different pHs

pH	H-1	H-3	H-2'	H-6'a	H-6'b	H-3''
3.01	3.65	3.61	3.68	3.31	3.45	3.52
4.21	3.64	3.61	3.68	3.31	3.45	3.52
5.71	3.62	3.49	3.55	3.28	3.45	3.51
7.33	3.38	3.25	3.35	3.2	3.43	3.38
8.81	3.04	3.01	3.13	5.95	3.31	3.10
10.13	2.92	2.86	2.96	2.83	2.98	3.03
11.00	2.92	2.86	2.96	2.83	2.98	3.03

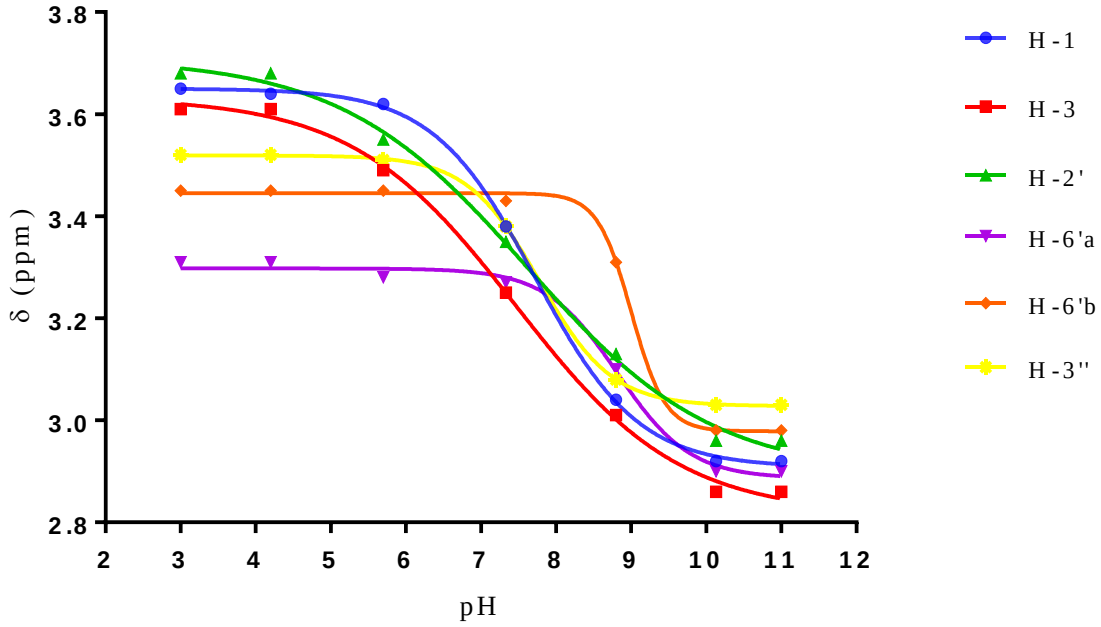


Fig. 4.5 NMR titration curves for the ^{1}H chemical shifts (δ) of 0.250-0.130 M tobramycin (5) were measured relative to TMSP in 100 % H_2O at 25°C

Table 4.5 pK_a values of individual nitrogen atoms of tobramycin in 99.97% D_2O and 100% H_2O, as indicated

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'	N-6'	N-3''
^{1}H NMR [a]	7.51 ±0.03	6.60 ±0.05	7.80 ±0.05	9.07 ±0.10 [c]	7.62 ±0.08
^{1}H NMR [b]	7.53	6.62	7.75	8.98 [d]	7.70

[a] This work in 99.97% D_2O

[b] This work in 100% H_2O

[c] The pK_a value of N-6' of tobramycin determined using ^{1}H NMR spectroscopy (in this work in 99.97% D_2O) is the average pK_a of the values of N-6' obtained using ^{1}H NMR spectroscopic data for 6'a (9.05) and 6'b (9.10)

[d] The pK_a value of N-6' of tobramycin determined using ^{1}H NMR spectroscopy (in this work in 100% H_2O) is the average pK_a of the values of N-6' obtained using ^{1}H NMR spectroscopic data for 6'a (8.90) and 6'b (9.05)

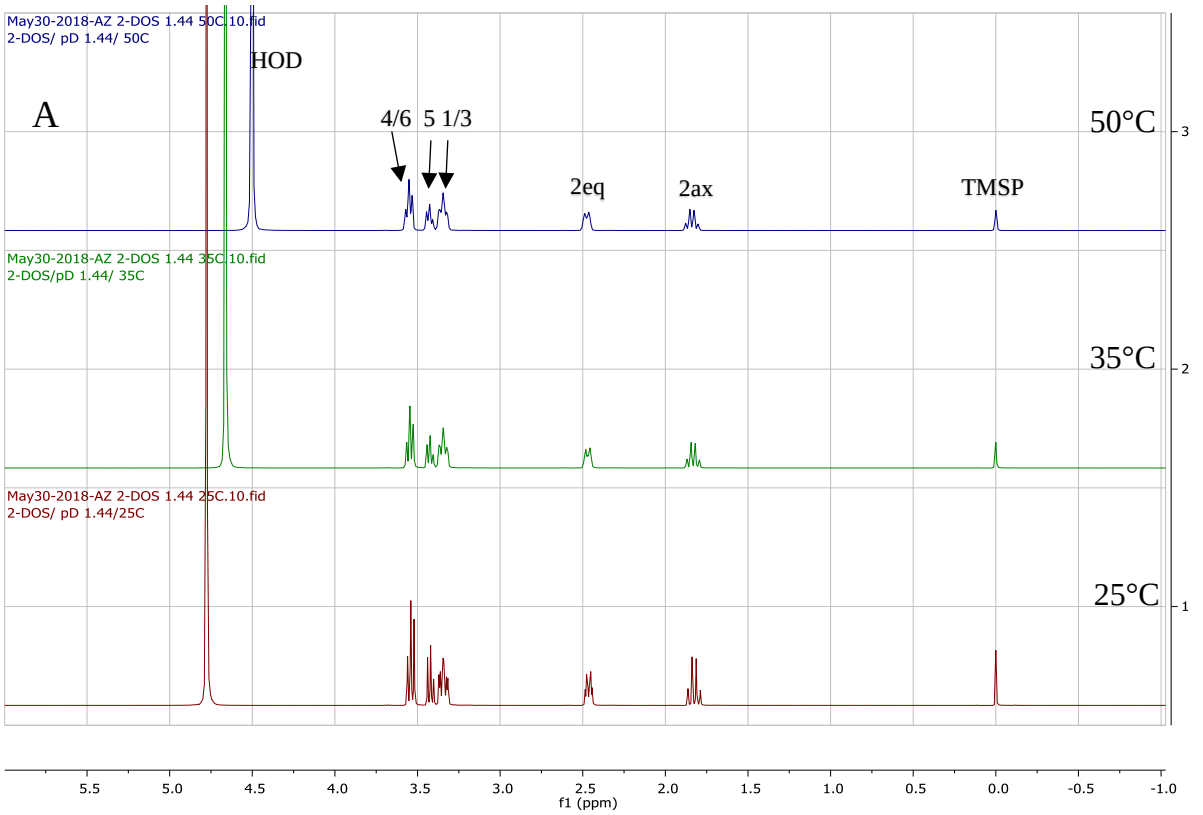
4.2. The effect of temperature and concentration on the pK_a values of aminoglycoside antibiotics

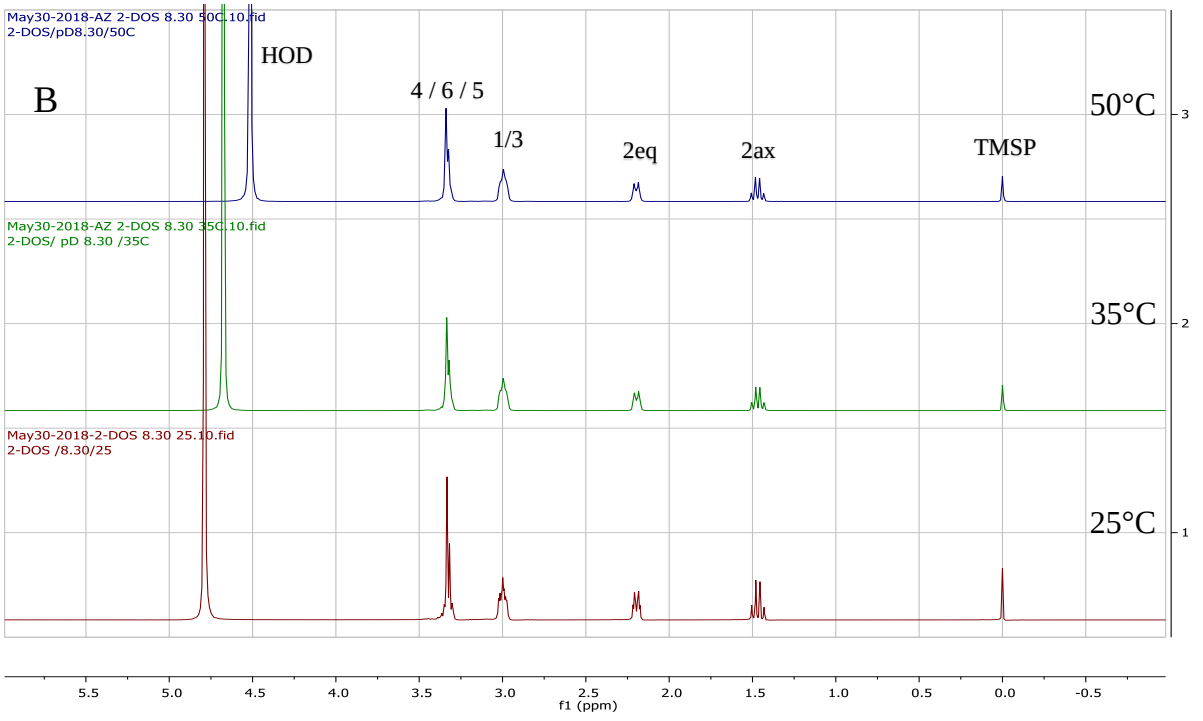
4.2.1. pD-dependent and temperature-dependent ^{1}H and ^{13}C NMR spectroscopic characterisations of 2-deoxystreptamine (1) and neomycin C (3)

^{1}H and ^{13}C NMR spectroscopic data were measured at pD 1.44, pD 8.30, and pD 11.68 for 2-deoxystreptamine and pD 1.25, pD 12.40 for neomycin, at three fixed temperature 25°C, 35°C, and 50°C. The results showed that the chemical shifts (δ) corresponding to the H-1/3 and C-1/3 of 2-deoxystreptamine (1) and the chemical shifts (δ) corresponding to the H-1, H-3, H-2',H-6', H-2''', H-6''' and C-1, C-3, C-2', C-6', C-2''', C-6''' of neomycin C (3) did not shift with the increasing temperature from 25°C to 50°C at low pD and high pD (see Figs. 4.6, 4.7 for 2-deoxystreptamine and Figs. 4.8, 4.9 for neomycin C). One possible explanation of this observation is that the chemical shifts (δ) of these protons and carbons of both 2-deoxystreptamine (1) and neomycin C (3) were not temperature dependent. Thus, the pK_a values of their amino groups on aminoglycosides will not be affected by increasing the temperature.

4.2.2. pD-dependent and concentration-dependent ^{1}H NMR spectroscopy characterisations of 2-deoxystreptamine (1) and neamine (2)

^{1}H NMR spectroscopic data were measured at two concentrations of 0.630 M and 0.157 M at low pD (~2) for 2-deoxystreptamine (1) and at 0.512 M and 0.128 M at high pD (~10) for neamine (2). The obtained results showed that the chemical shifts (δ) corresponding to the H-1/3 of 2-deoxystreptamine (1) and the chemical shifts (δ) corresponding to the H-1, H-3, H-2', and H-6' of neamine (2) did not shift with the changing concentration levels. Thus, the pK_a values of N-1/3 on 2-deoxystreptamine and N-1, N-3, N-2', and N-6' on neamine will not be affected by changing their concentrations, at least in this typical NMR concentration range (see Fig. 4.10 for 2-deoxystreptamine and Fig. 4.11 for neamine).


May30-2018-AZ 2-DOS 1.44 50C.10.fid
2-DOS/ pD 1.44/ 50C
HOD
A
4/6
5 1/3
2eq
2ax
TMSP
50°C
May30-2018-AZ 2-DOS 1.44 35C.10.fid
2-DOS/pD 1.44/ 35C
35°C
May30-2018-AZ 2-DOS 1.44 25C.10.fid
2-DOS/ pD 1.44/25C
25°C
f1 (ppm)


May30-2018-AZ 2-DOS 8.30 50C.10.fid
2-DOS/pD8.30/50C
HOD
B
4 / 6 / 5
1/3
2eq
2ax
TMSP
50°C
May30-2018-AZ 2-DOS 8.30 35C.10.fid
2-DOS/ pD 8.30 /35C
35°C
May30-2018-2-DOS 8.30 25.10.fid
2-DOS /8.30/25
25°C
f1 (ppm)

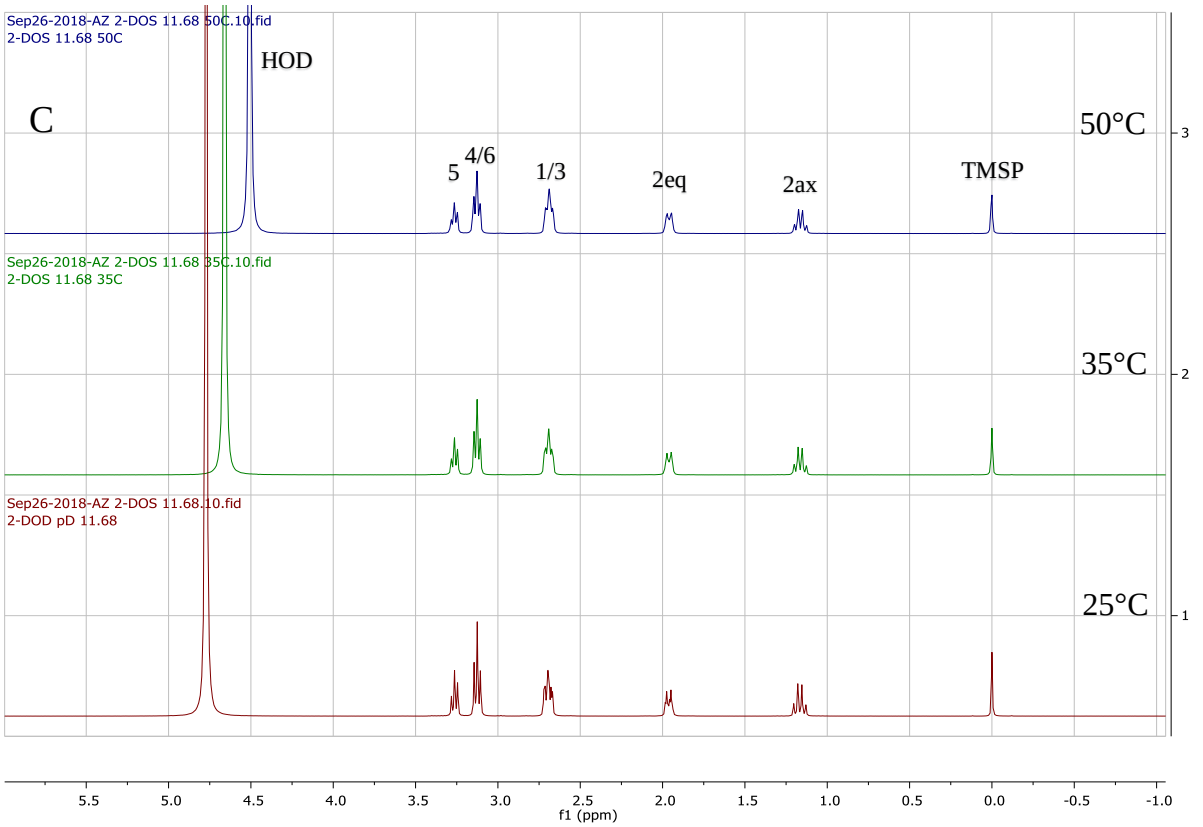
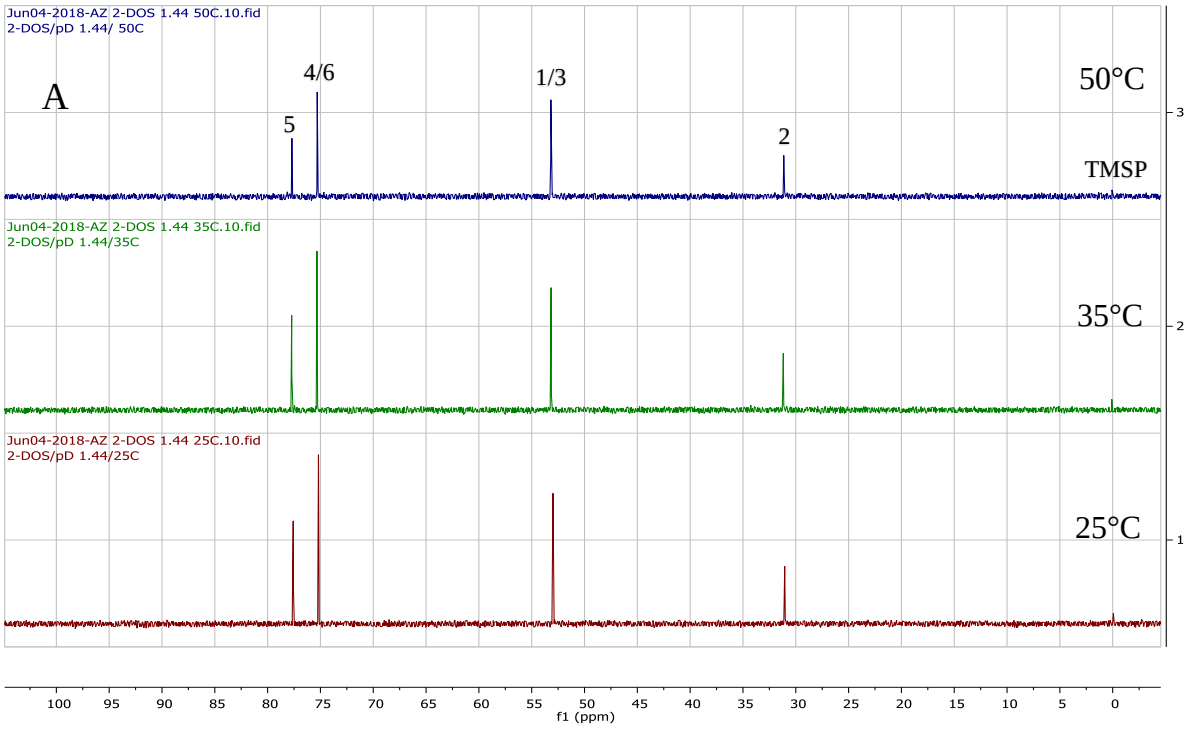


Fig. 4.6 ^{1}H NMR spectra of 0.159 M 2-deoxystreptamine (1) were measured relative to TMSP in 99.97% D_2O at 25°C (red), 35°C (green), and 50°C (blue), A) at pD 1.44, B) at pD 8.30, and C) at pD 11.68



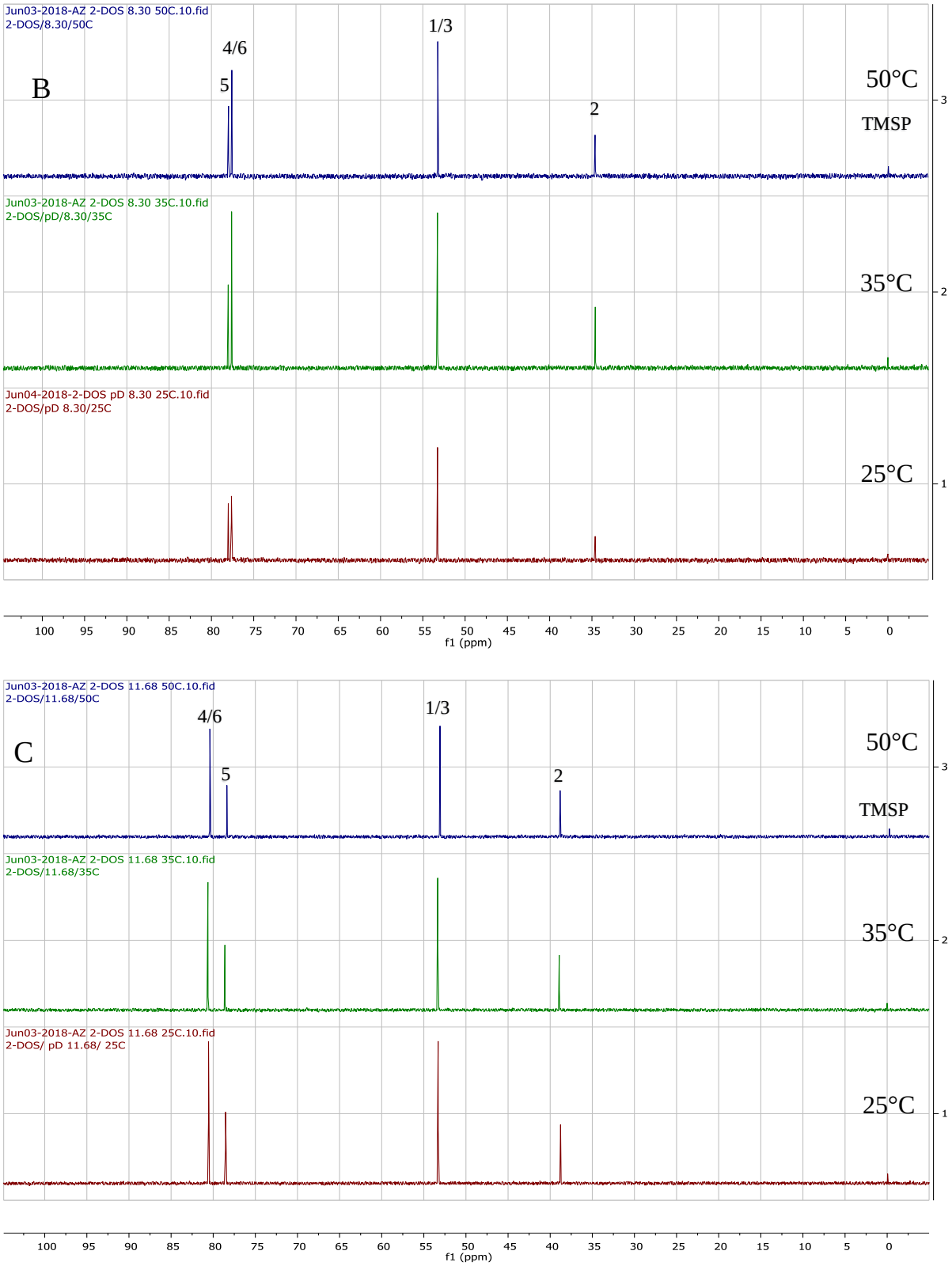
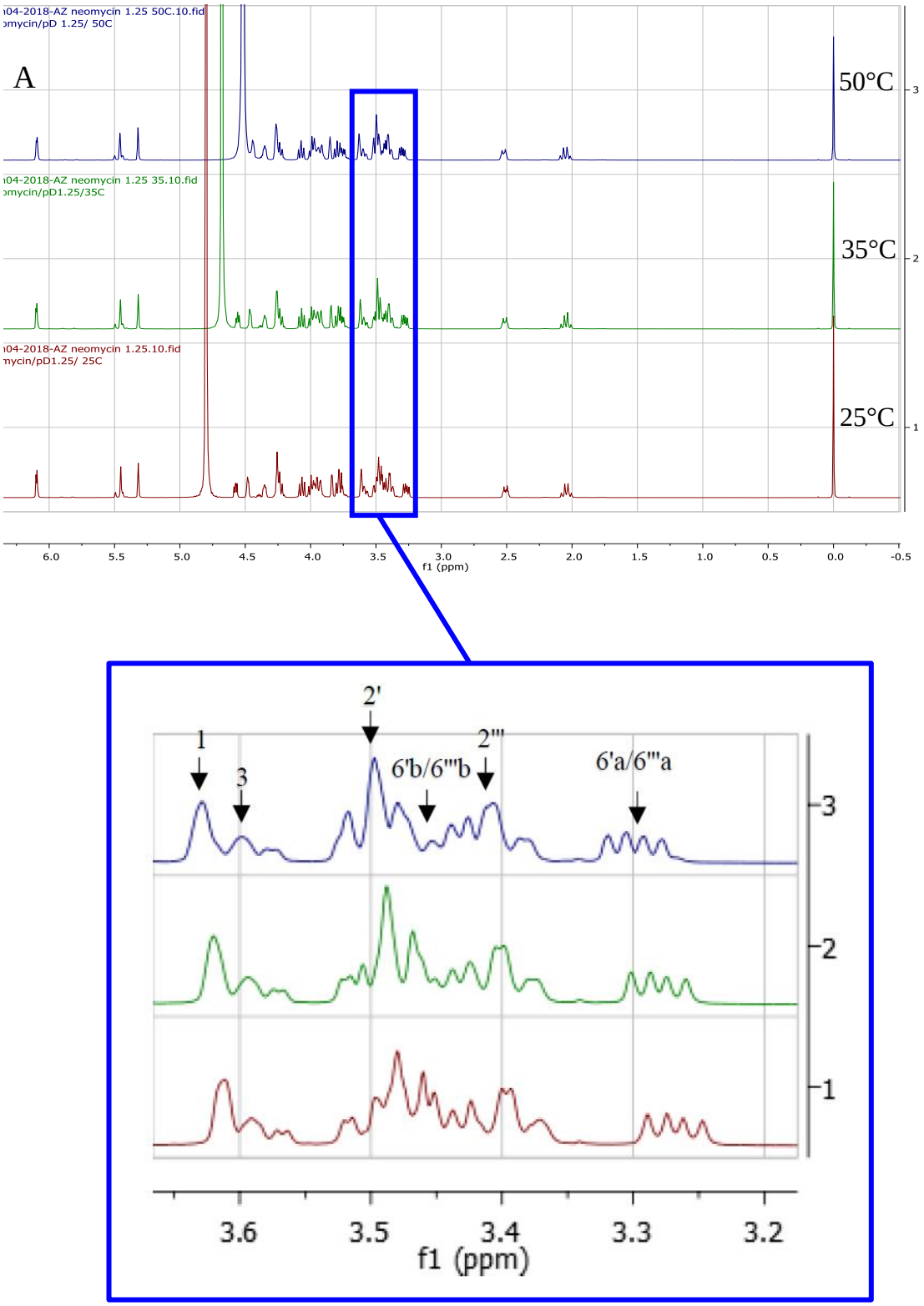


Fig 4.7 ^{13}C NMR spectra of 0.159 M 2-deoxystreptamine (1) were measured relative to TMSP in 99.97% D_2O at 25°C (red), 35°C (green), and 50°C (blue), A) at pD 1.44, B) at pD 8.30, and C) at pD 11.68


A
50°C
35°C
25°C
f1 (ppm)
1
3
2'
6'b/6'''b
2'''
6'a/6'''a
3.6
3.5
3.4
3.3
3.2
f1 (ppm)

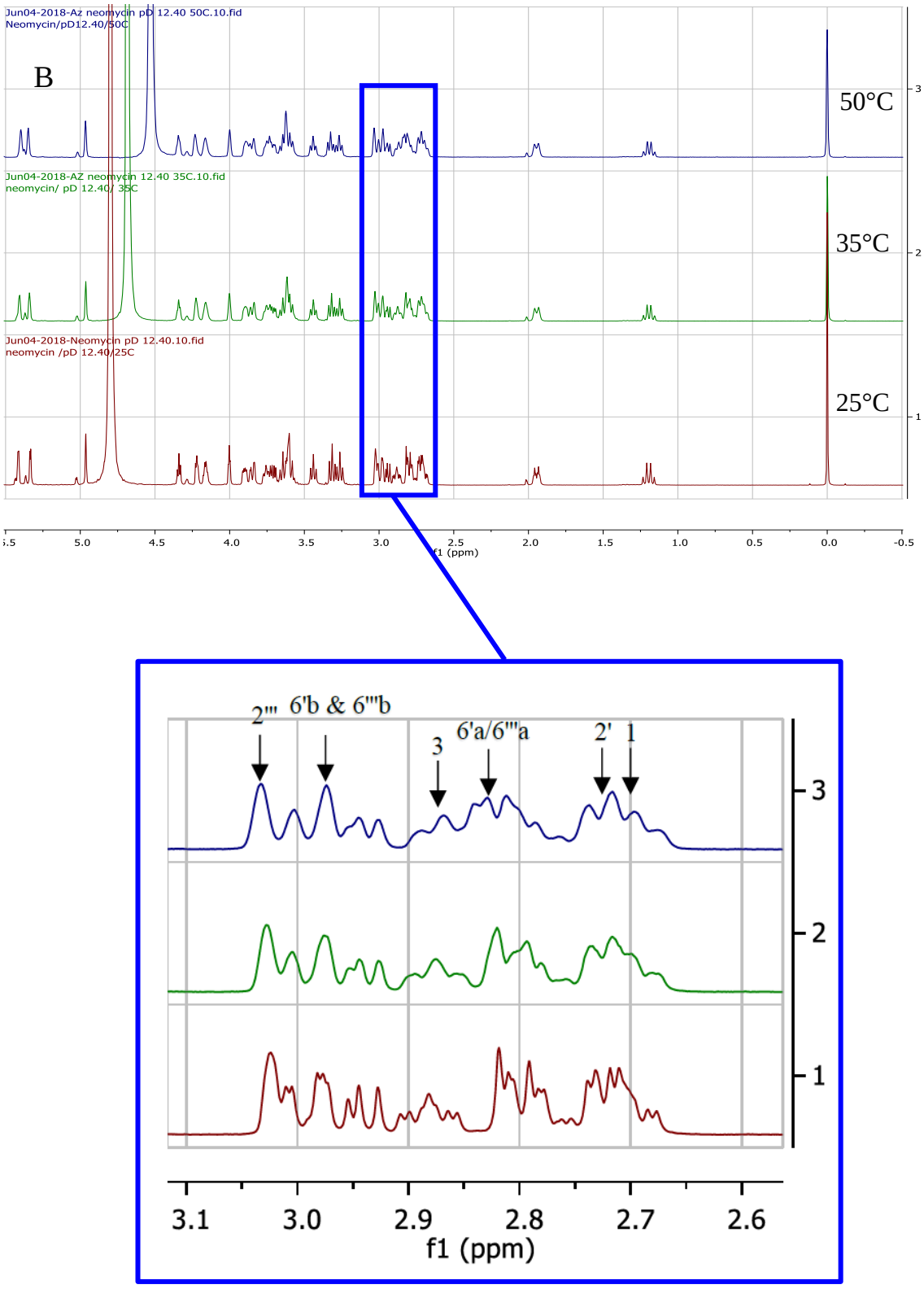
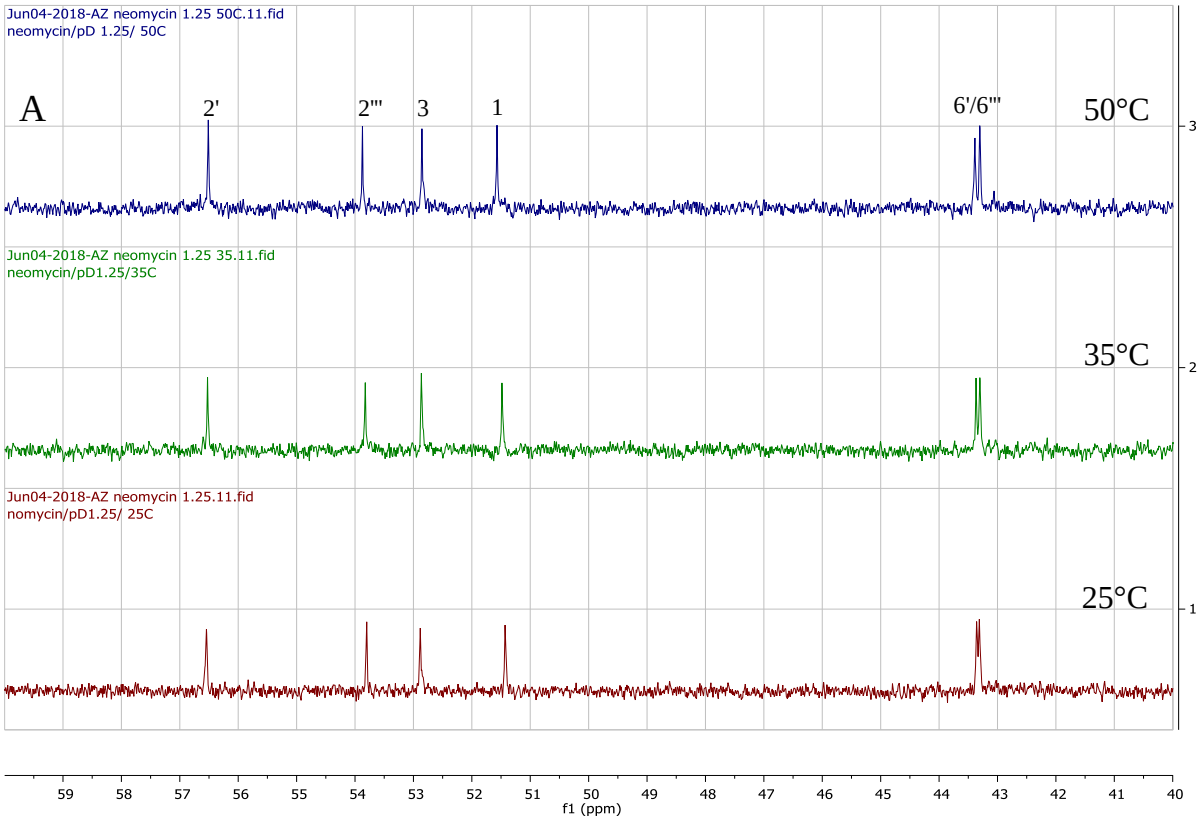


Fig. 4.8 ^{1}H NMR spectra of 0.090 M neomycin C (3) were measured relative to TMSP in 99.97% D_2O at 25°C (red), 35°C (green), and 50°C (blue), A) at pD of 1.25 and B) at pD of 12.40



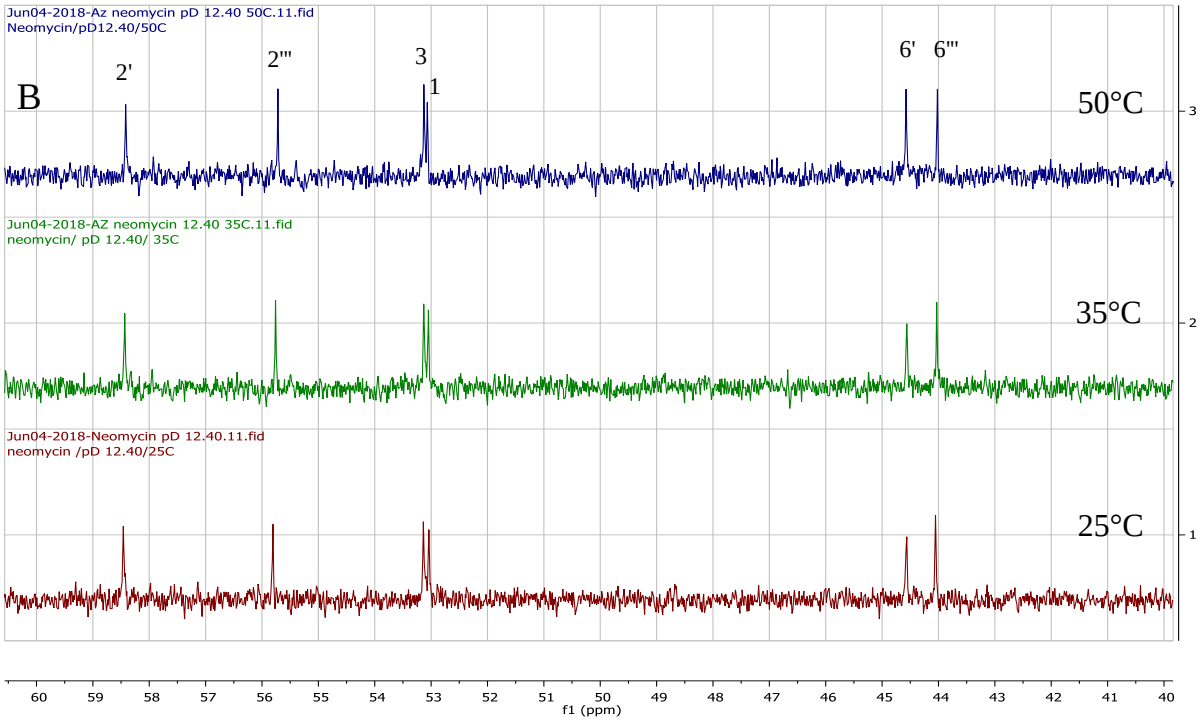


Fig. 4.9 ^{13}C NMR spectra of 0.090 M neomycin C (3) were measured relative to TMSP in 99.97% D_2O at 25°C (red), 35°C (green), and 50°C (blue), A) at pD of 1.25 and B) at pD of 12.40

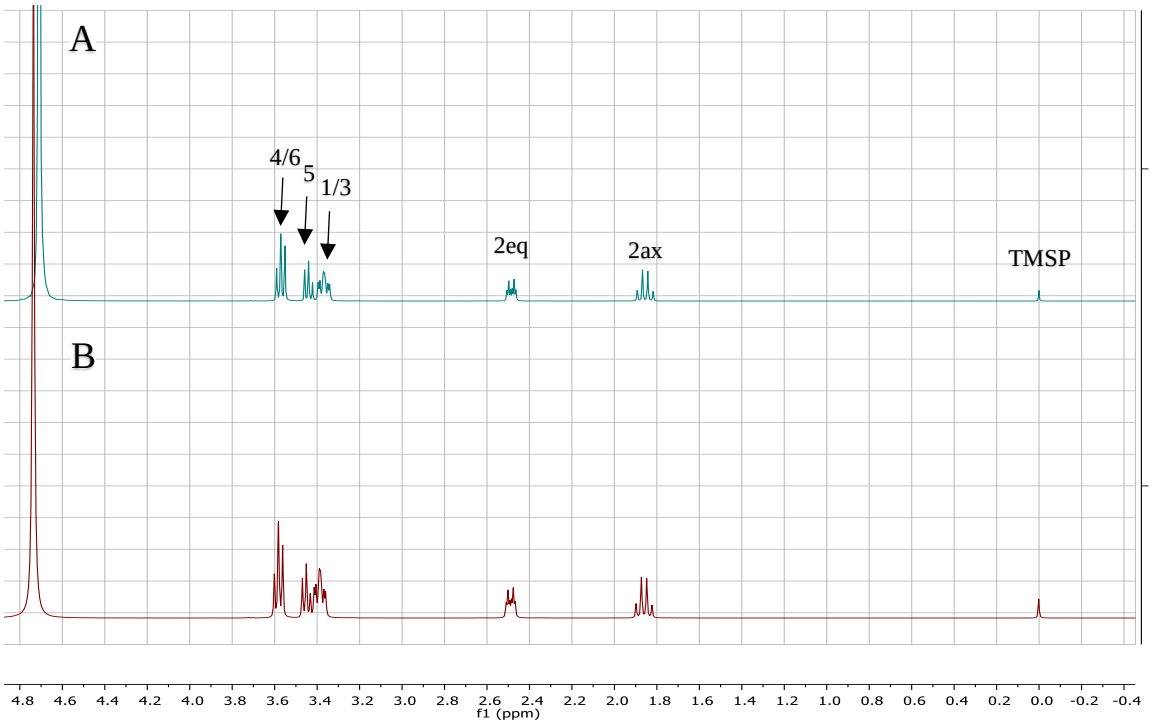


Fig. 4.10 ^{1}H NMR spectra of 2-deoxystreptamine (1) were measured relative to TMSP in 99.97% D_2O at 25°C, A) at concentration of 0.630 M and pD 1.88, B) at concentration of 0.157 M and pD 2.00

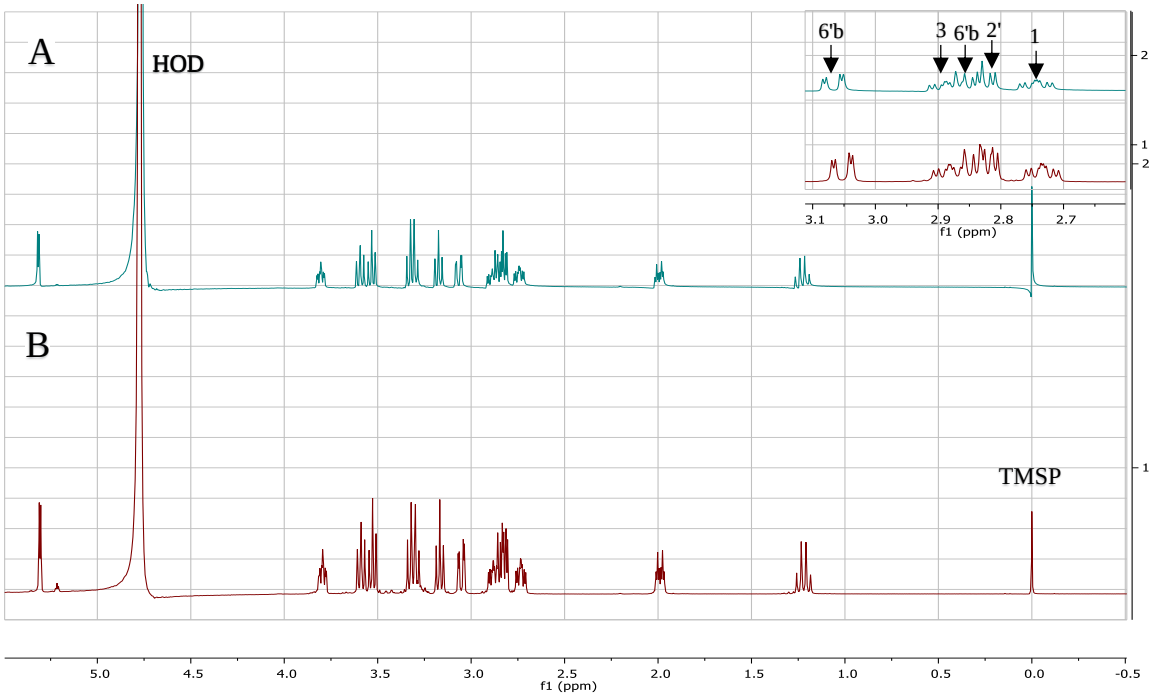


Fig. 4.11 ^{1}H NMR spectra of neamine (2) were measured relative to TMSP in 99.97% D_2O at 25°C, A) at concentration of 0.512 M and pD 10.00, B) at concentration of 0.128 M and pD 10.10

4.3. Determination of the pK_a values of the individual amino groups on aminoglycosides using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy in D_2O

The sections 4.3.1 to 4.3.10 present the pK_a values of the individual amines on aminoglycosides determined using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy in this work. These data are compared with the published data (see Tables 4.9 for 2-deoxystreptamine, 4.13 for neamine, 4.17 for neomycin C, 4.21 for paromomycin, 4.25 for tobramycin, 4.29 for kanamycin B, 4.33 for netilmicin, 4.37 for sisomicin,4.41 for amikacin, and 4.45 for streptomycin).

The ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopic experiments of 2-deoxystreptamine were repeated 3 times. Similarly, each NMR experiment was repeated twice for neamine, neomycin C, and tobramycin in order to calculate the error bars (Standard Deviation, SD) for the pH values, the chemical shifts (δ), and the pK_a values (make 3, for 2-deoxystreptamine, or 2, for neamine, neomycin C, and tobramycin, samples of the same compound at the same pH and measure the NMR). It was observed that the majority of the error bars for the pH values and for the chemical shifts (δ) were the same size as the plotted points (symbols) on the nonlinear sigmoidal curves. Therefore, having determined by experiments the typical size of the error bars, n=1 was judged to be sufficient for obtaining further NMR spectroscopic data.

4.3.1. pK_a values of the individual amino groups of 2-deoxystreptamine (1)

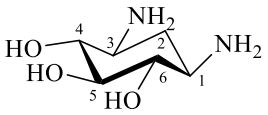


Fig. 4.12 2-Deoxystreptamine (1)

2-Deoxystreptamine (Fig. 4.12) is substituted with two primary amines on a cyclohexane ring. It is the central moiety of neamine, neomycin C, paromomycin, tobramycin, kanamycin B, netilmicin, sisomicin, and amikacin (see Fig. 4.1). The pK_a determination using ^{1}H, ^{13}C, and

^{15}N HMBC NMR were repeated 3 times (see Tables 4.6a, b, c, 4.7a, b, c, and 4.8a, b, c for ^{1}H, ^{13}C and ^{15}N chemical shifts, respectively). Tables 4.6d, 4.7d, and 4.8d show the average of Tables 4.6a, b, c, 4.7a, b, c, and 4.8a, b, c for ^{1}H, ^{13}C, and ^{15}N chemical shifts, respectively (all the tables are shown in the appendix). The nonlinear sigmoidal curves are shown in Figs. 4.13a, b, 4.14a, b, 4.15, 4.16, 4.17, and 4.18. The pK_a values of the two individual nitrogen atoms of 2-deoxystreptamine, shown in Table 4.9 and Fig. 4.19, were extracted from the inflection points of the sigmoidal curves.

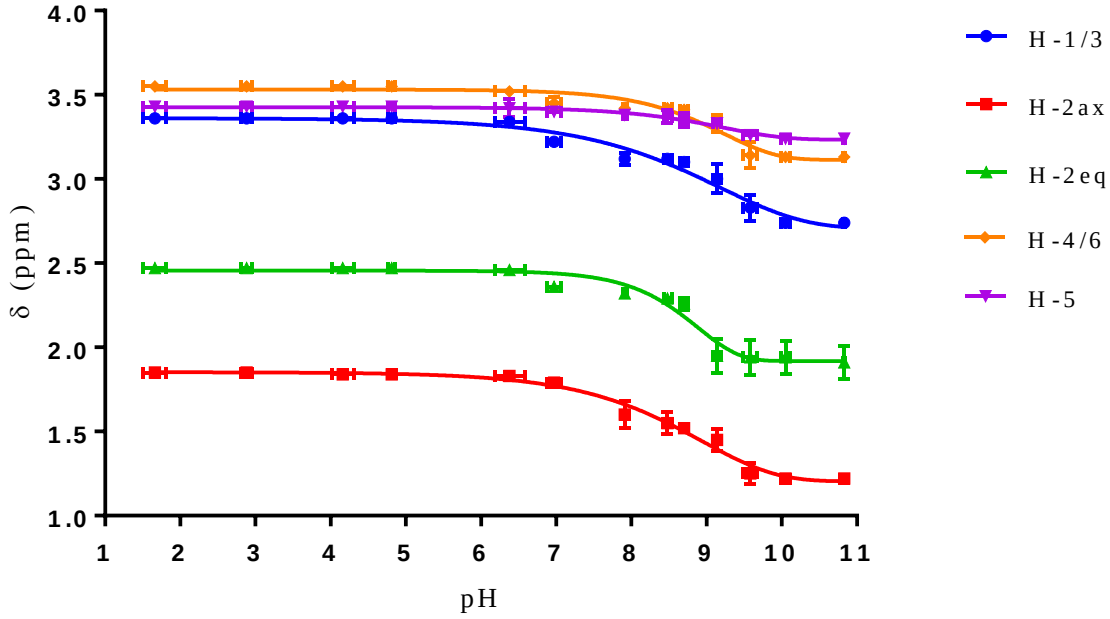


Fig. 4.13a NMR titration curves for the ^{1}H chemical shifts (δ) (500 MHz) of 0.243-0.111 M 2-deoxystreptamine were measured relative to TMSP in 99.97% D_2O at 25°C

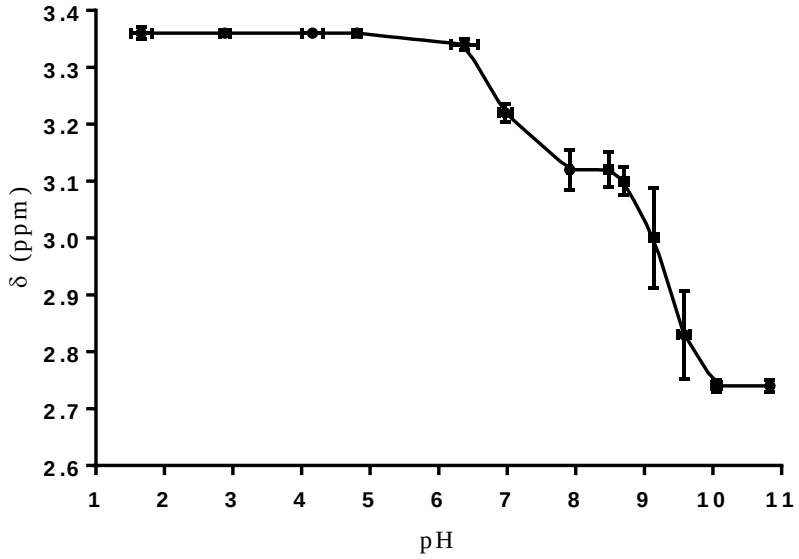


Fig. 4.13b NMR titration curve of H-1 and H-3, expanded from Fig. 4.13a.

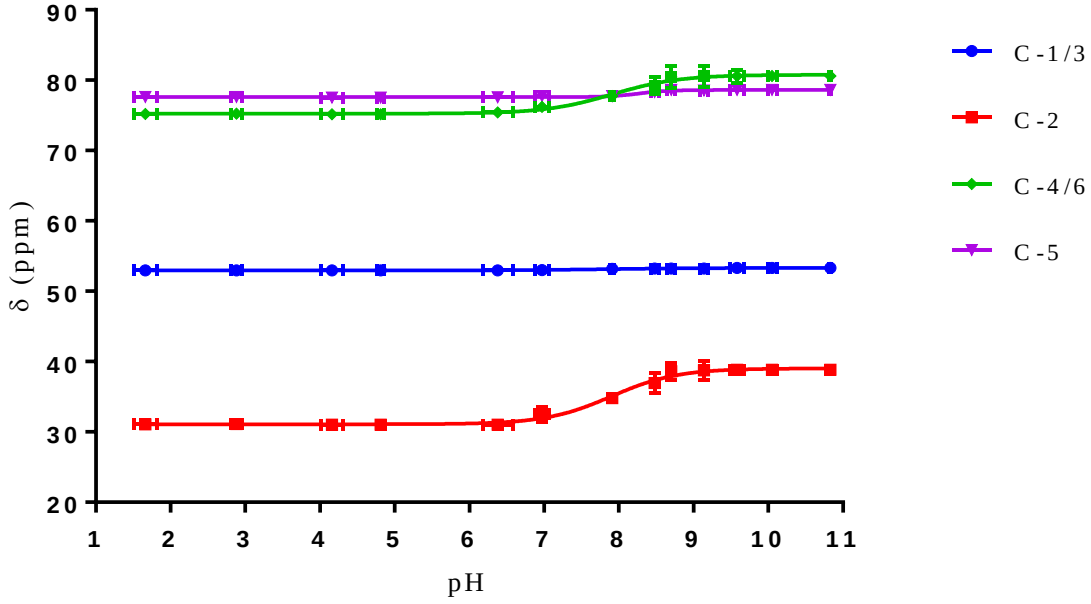


Fig. 4.14a NMR titration curves for the ^{13}C chemical shifts (δ) (125.77 MHz) of 0.243-0.111 M 2-deoxystreptamine were measured relative to TMSP in 99.97% D_2O at 25°C

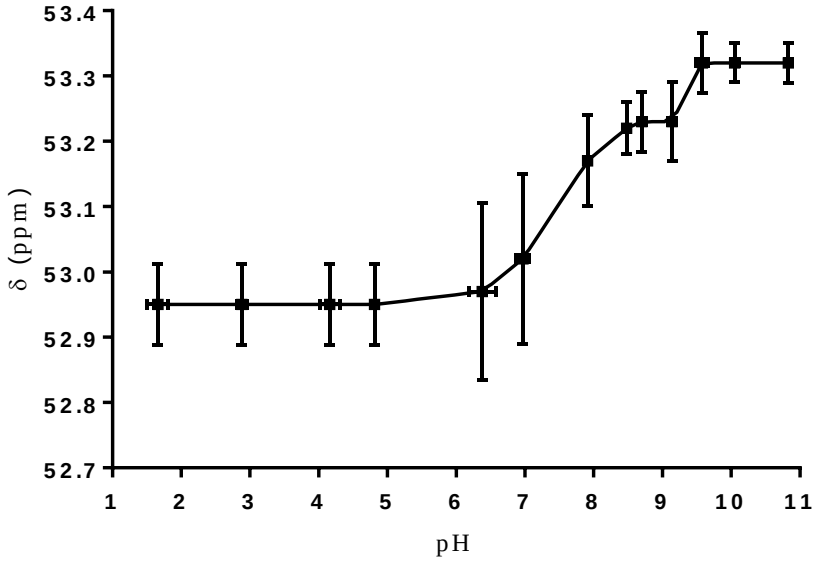


Fig. 4.14b NMR titration curve of C-1 and C-3, expanded from Fig. 4.14a

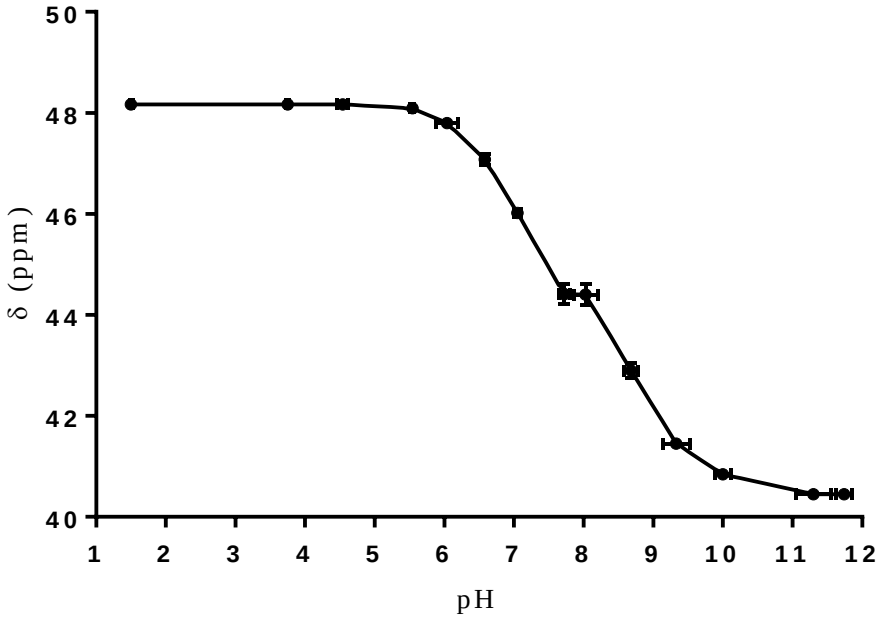
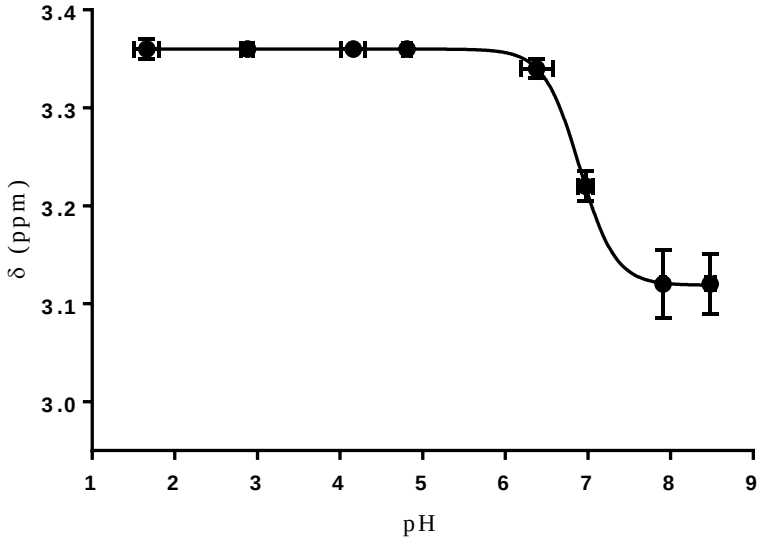


Fig. 4.15 NMR titration curves for the ^{15}N HMBC chemical shifts (δ) (50.67 MHz) of 0.631-0.369 M 2-deoxystreptamine were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C

It can be easily seen that the two inflections on the sigmoidal curves of H-3, H-1, and C-3, C-1, and N-3, N-1, as shown in Figs. 4.13b, 4.14b, and 4.15, respectively, are not best-

fitted curves, they are shown as point-joining curves. However, there is no available choice to draw best-fitted curves of multiple inflections curves (in these case each line has two inflections) using GraphPad Prism. For this reason, the two inflections on the sigmoidal curves of H-3, H-1, and C-3, C-1, and N-3, N-1 have been separated and drawn separately as lines of best-fit (see Figs. 4.16 and 4.17 and 4.18).

A



B

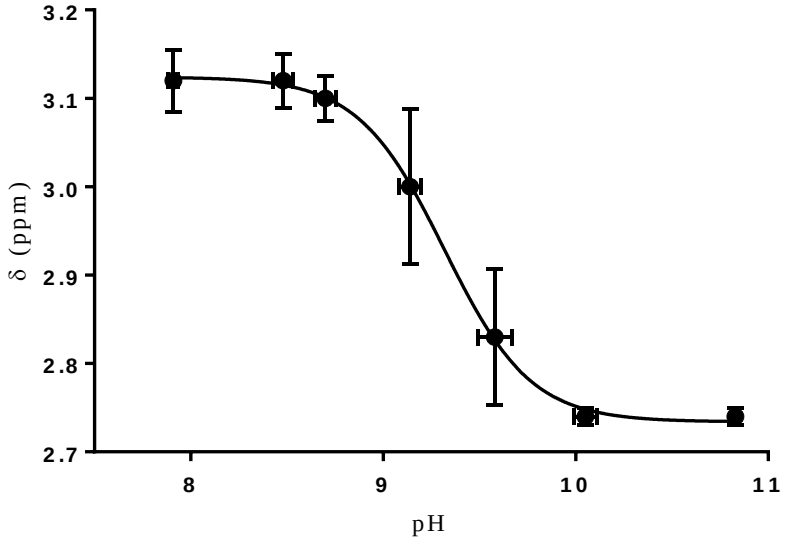
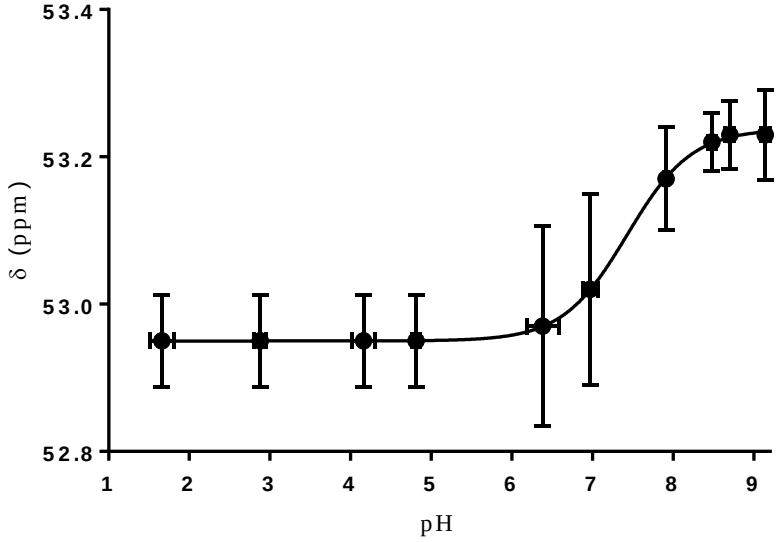


Fig. 4.16 NMR titration curves of A) H-3 and B) H-1, expanded from Fig. 4.13b

A



B

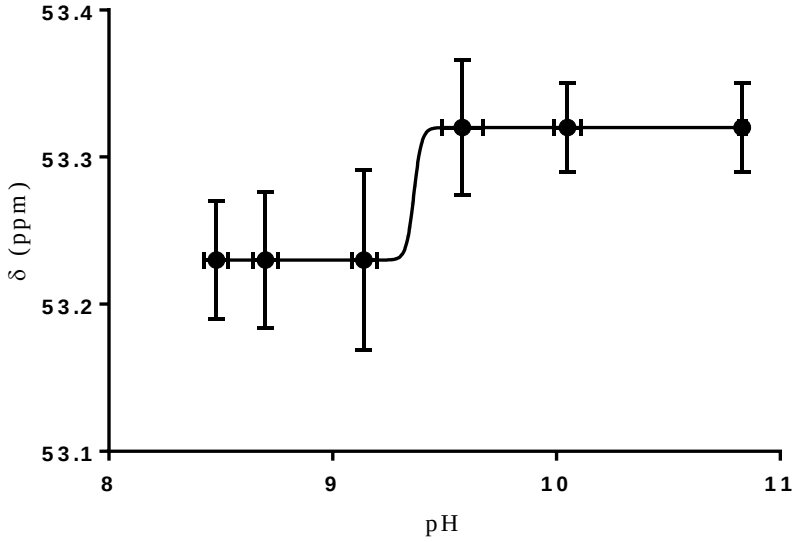
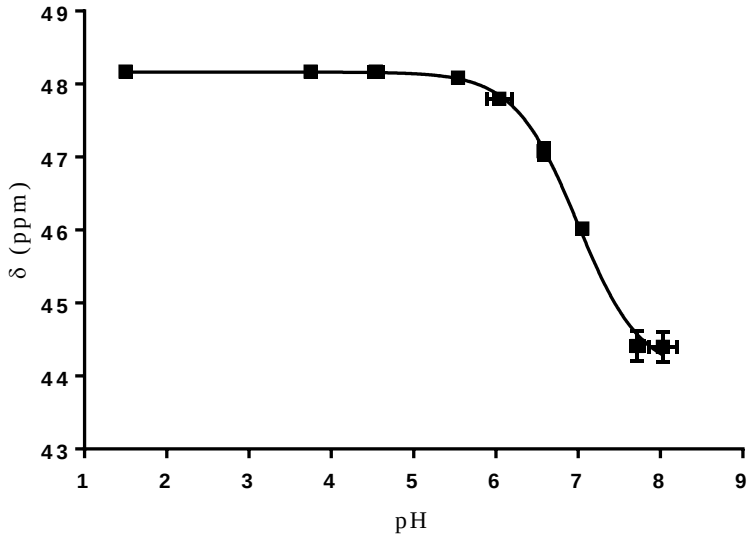


Fig. 4.17 NMR titration curves of A) C-3 and B) C-1, expanded from Fig. 4.14b

A



B

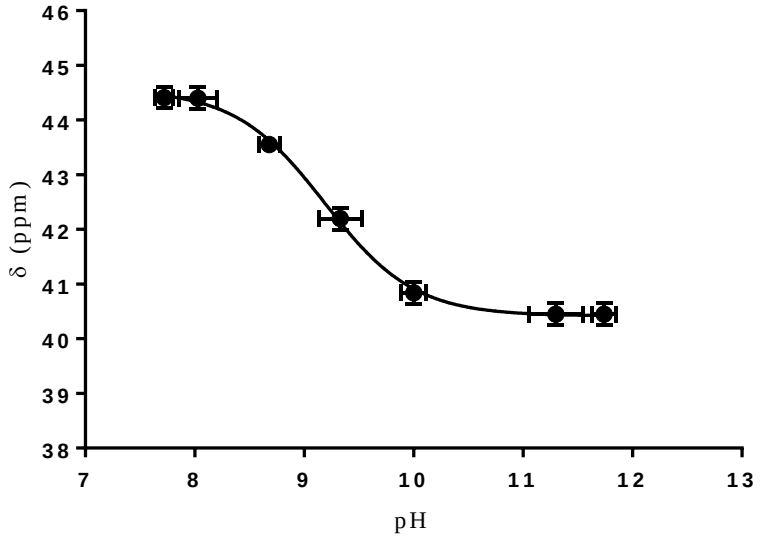


Fig. 4.18 NMR titration curves of A) N-3 and B) N-1, expanded from Fig. 4.15

Table 4.9 pK_a values of individual nitrogen atoms of 2-deoxystreptamine determined using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy in this work

Individual nitrogen atoms pK_a / Method	N-1	N-3
^{1}H NMR [b]	9.25 ± 0.05	6.97 ± 0.04
^{13}C NMR [b]	9.35 ± 0.10	7.03 ± 0.05
^{15}N HMBC NMR [b]	9.20 ± 0.05	7.01 ± 0.05

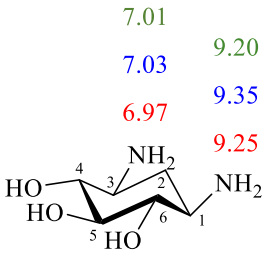


Fig. 4.19 pK_a values of individual nitrogen atoms of 2-deoxystreptamine determined using ^{1}H (red), ^{13}C (blue), and ^{15}N HMBC (green) NMR spectroscopy

Due to the symmetrical character of 2-deoxystreptamine, the signals of H-1/3, C-1/3, and N-1/3 have same chemical shifts (δ). Therefore, there are two inflections in the H-1/3, C-1/3, and N-1/3 sigmoidal curves. The inflection point of each inflection represent a pK_a value (see Figs. 4.13b, 4.14b, 4.15, and Fig. 4.20).

In this work, the amines on 2-deoxystreptamine were numbered by comparing them to the 2-deoxystreptamine ring of neamine (2), neomycin C (3), paromomycin (4), tobramycin (5), kanamycin B (6), netilmicin (7), and sisomicin (8).

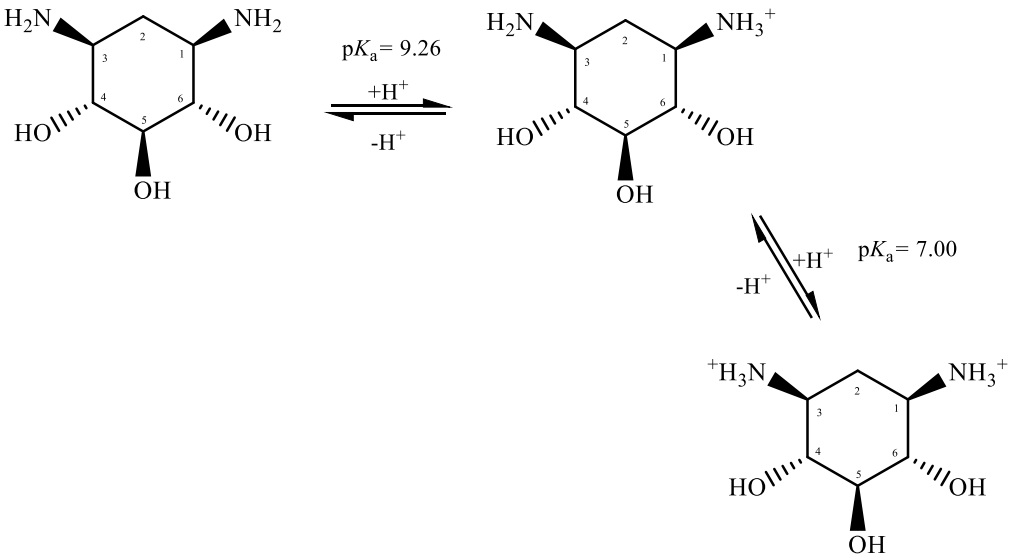


Fig. 4.20 Protonation scheme of 2-deoxystreptamine (1) containing two amino groups.

4.3.2. pK_a values of the individual amino groups of neamine (2)

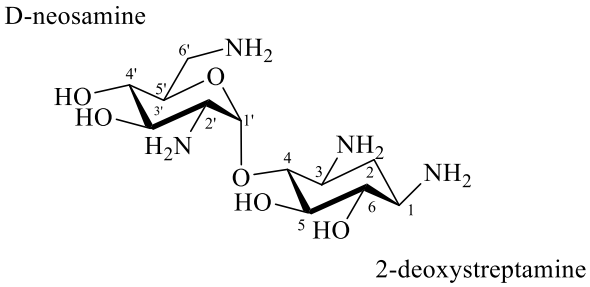


Fig. 4.21 Neamine (2)

Neamine includes four primary amines around two rings, one of which is 2-deoxy-streptamine, the other is a D-sugar on a glucose hexose template (see Fig. 4.21). All the pK_a determinations using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy were repeated twice. The average of the chemical shifts of ^{1}H, ^{13}C, and ^{15}N HMBC of neamine at different pHs, shown in Tables 4.10, 4.11, and 4.12, were plotted against the pH values of the solution (all the tables are shown in the appendix). The nonlinear sigmoidal curves are shown in Figs. 4.22, 4.23, and 4.24. The pK_a values of each individual nitrogen atom of neamine, shown in Table 4.13 and Fig. 4.25, were then extracted from the inflection points of the sigmoidal curves.

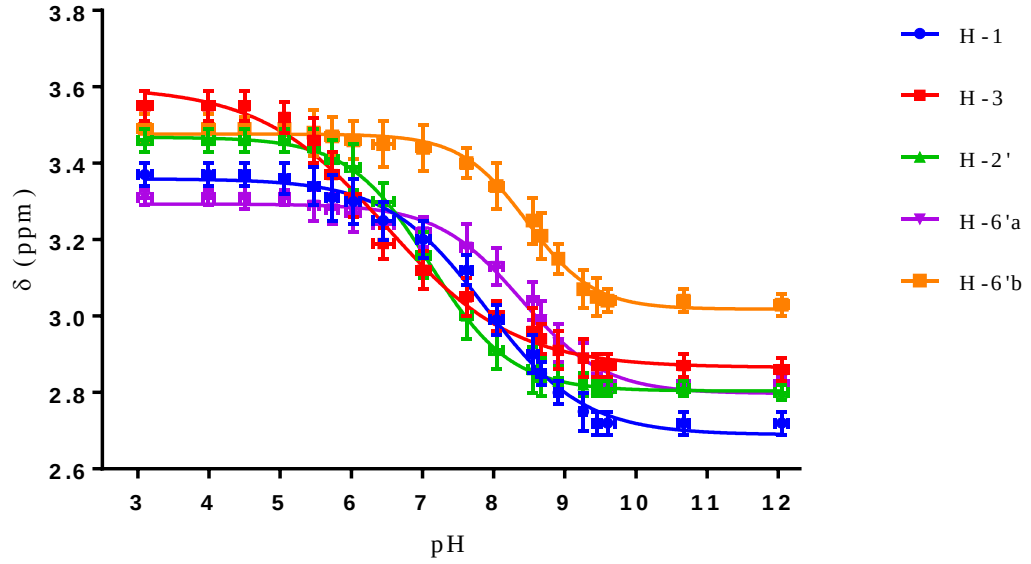


Fig. 4.22 NMR titration curves for the ^{1}H chemical shifts (δ) (500 MHz) of 0.243-0.155 M neamine were measured relative to TMSP in 99.97% D_2O at 25°C

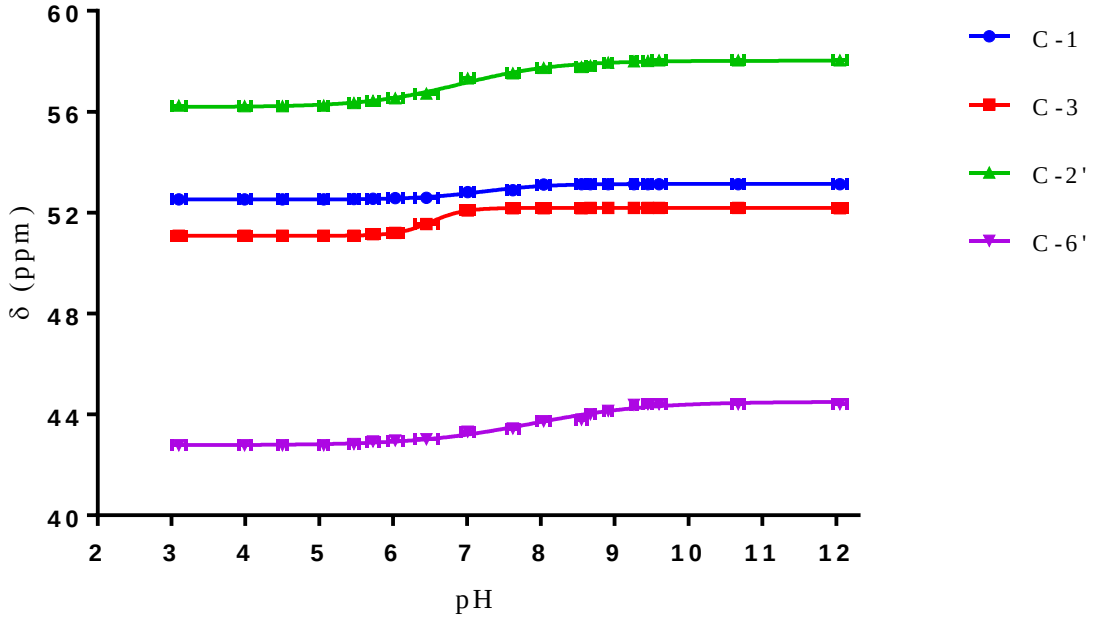


Fig. 4.23 NMR titration curves for the ^{13}C chemical shifts (δ) (125.77 MHz) of 0.243-0.155 M neamine were measured relative to TMSP in 99.97% D_2O at 25°C

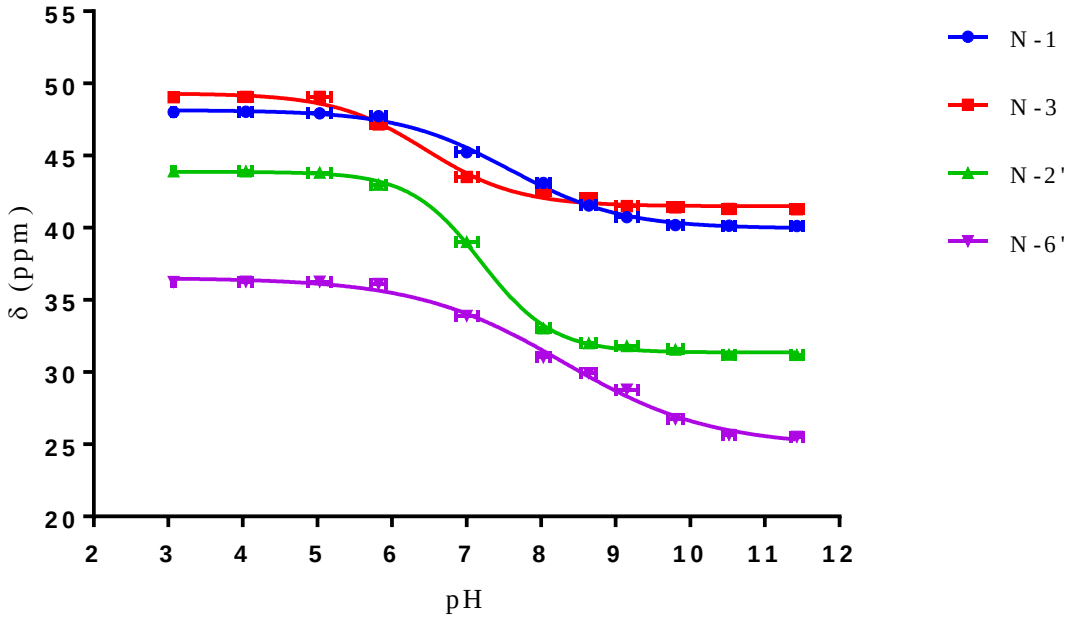


Fig. 4.24 NMR titration curves for the ^{15}N HMBC chemical shifts (δ) (50.67 MHz) of 0.243-0.155 M neamine were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C

Table 4.13 pK_a values of individual nitrogen atoms of neamine determined using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy in this work and then compared with the published data, as indicated

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'	N-6'
^{1}H NMR [a]	7.77	6.44	7.23	8.08
^{1}H NMR [b]	7.62 ± 0.03	6.50 ± 0.04	7.10 ± 0.05	8.34 ± 0.05 [c]
^{13}C NMR [b]	7.60 ± 0.05	6.55 ± 0.05	7.05 ± 0.05	8.25 ± 0.05
^{15}N HMBC NMR [b]	7.60 ± 0.05	6.50 ± 0.05	7.20 ± 0.10	8.35 ± 0.05

[a] pK_a values of individual nitrogen atoms of neamine determined using ^{1}H NMR spectroscopy in D_2O relative to the HDO peak at 25°C (Andac et al., 2011)

[b] This work

[c] The pK_a value of N-6' of neamine determined using ^{1}H NMR spectroscopy (in this work) is the average pK_a of the values of N-6' obtained using ^{1}H NMR spectroscopic data for H-6'a (8.33) and 6'b (8.35)

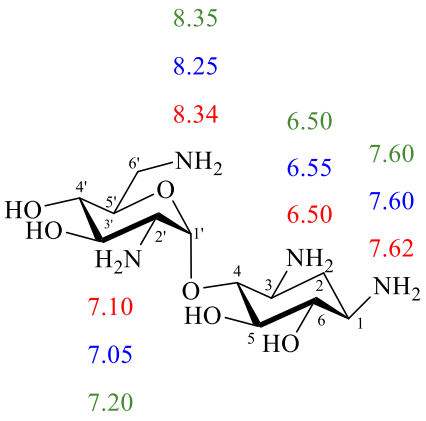


Fig. 4.25 pK_a values of individual nitrogen atoms of neamine determined using ^{1}H (red), ^{13}C (blue), and ^{15}N HMBC (green) NMR spectroscopy

After calculating the average pK_a values, using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopic data, for each individual nitrogen atom on neamine are: N-1 = 7.60, N-3 = 6.51, N-2' = 7.11, and N-6' = 8.31. The assignment order of the average ionisation constants is: N-6' > N-1 > N-2' > N-3. These pK_a values are consistent in magnitude and in assignment order with these reported in the literature (Andac et al., 2011).

4.3.3. pK_a values of the individual amino groups of neomycin C (3)

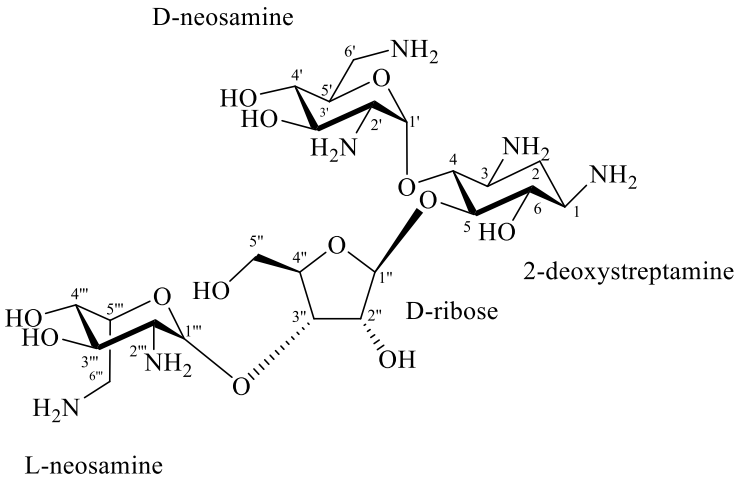


Fig. 4.26 Neomycin C (3)

Neomycin C has six primary amines. Those amines are substituents on two aminosugar rings: D-neosamine and L-neosamine and a central cyclohexane ring (2-deoxystreptamine). These three 6-membered rings are themselves O-substituents pendant from a D-ribose furanose ring. Neomycin C is a 4,5-*O*-disubstituted 2-deoxystreptamine (see Fig. 4.26). The pK_a determinations using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy were repeated twice. The average of the chemical shifts of ^{1}H, ^{13}C, and ^{15}N HMBC of neomycin at different pHs, shown in Tables 4.14, 4.15, and 4.16, were plotted against the pH values of the solution (all the tables are shown in the appendix). The nonlinear sigmoidal curves are shown in Figs. 4.27, 4.28, and 4.29. The pK_a values of individual nitrogen atoms of neomycin C, shown in Table 4.17 and Fig. 4.30, were extracted from the inflection points of the sigmoidal curves.

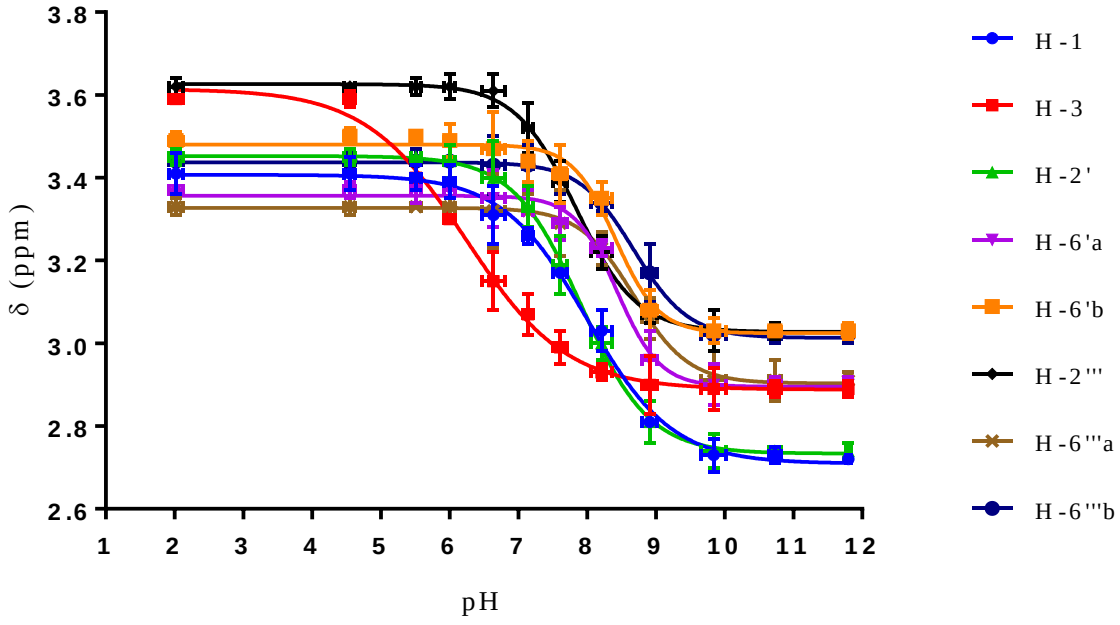


Fig. 4.27 NMR titration curves for the ^{1}H chemical shifts (δ) (500 MHz) of 0.218-0.122 M neomycin C were measured relative to TMSP in 99.97% D_2O at 25°C

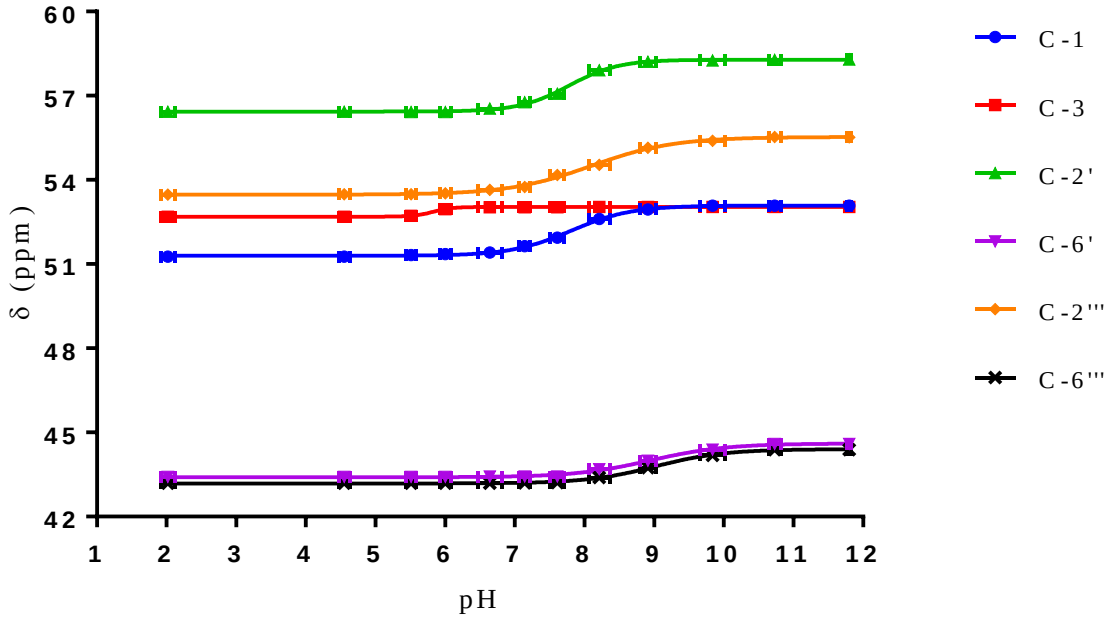


Fig. 4.28 NMR titration curves for the ^{13}C chemical shifts (δ) (125.77 MHz) of 0.218-0.122 M neomycin C were measured relative to TMSP in 99.97% D_2O at 25°C

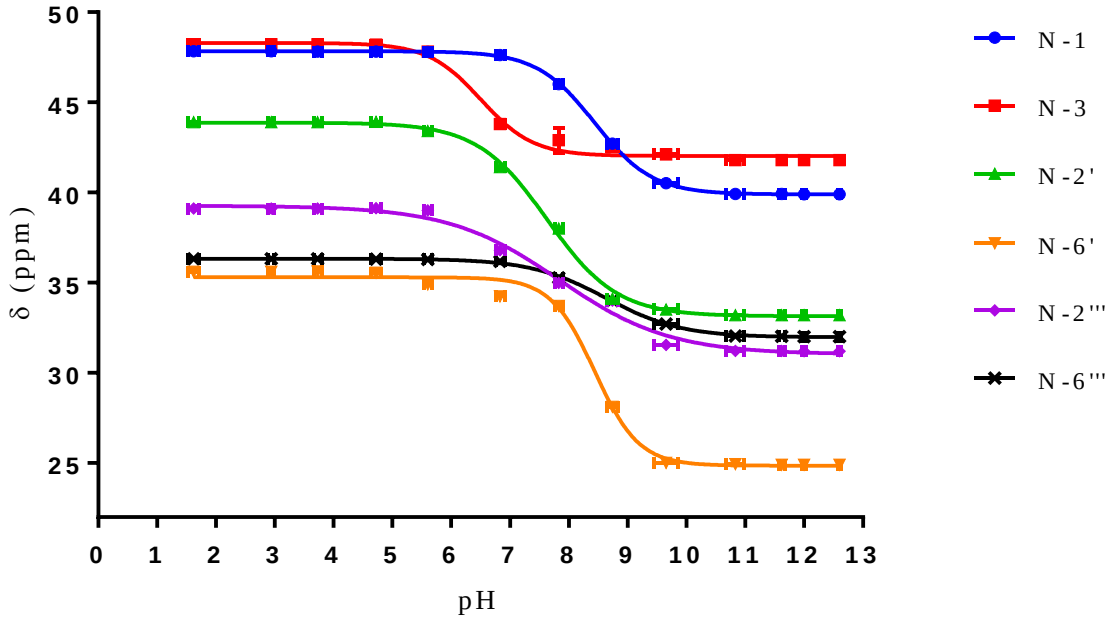


Fig. 4.29 NMR titration curves for the ^{15}N HMBC chemical shifts (δ) (50.67 MHz) of 0.392-0.218 M neomycin C were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C

Table 4.17 pK_a values of individual nitrogen atoms of neomycin C determined using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy and then compared with the published data, as indicated

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'	N-6'	N-2'''	N-6'''
^{15}N NMR [a]	7.90	5.70	7.40	8.10	7.70	8.70
^{15}N NMR [b]	8.04	5.74	7.55	8.60	7.60	8.80
^{15}N NMR [c]	8.70	6.90	8.20	9.20	8.30	9.50
^{1}H-^{13}C HSQC NMR [d]	8.10	5.40	7.40	8.70	7.50	8.80
^{1}H NMR [e]	8.10 ±0.07	6.90 ±0.02	8.00 ±0.05	8.67 ±0.05 [f]	8.05 ±0.05	8.73 ±0.05 [f]
^{13}C NMR [e]	8.00 ±0.05	6.90 ±0.05	8.00 ±0.05	8.70 ±0.04	8.15 ±0.06	8.86 ±0.10
^{15}N HMBC NMR [e]	8.15 ±0.10	6.80 ±0.05	7.95 ±0.07	8.60 ±0.05	7.90 ±0.10	8.71 ±0.07

[a] pK_a values of individual nitrogen atoms of neomycin determined using ^{15}N NMR spectroscopy in H_2O/D_2O (90: 10 v/v) relative to $^{15}NH_4Cl$ at 25°C (Özen et al., 2006)

[b] pK_a values of individual nitrogen atoms of neomycin determined using ^{15}N NMR spectroscopy in H_2O/D_2O (85: 15 v/v) relative to $NH_4{}^{15}NO_3$ at 25°C (Botto and Coxon, 1982)

[c] pK_a values of individual nitrogen atoms of neomycin determined using ^{15}N NMR spectroscopy in H_2O/D_2O (90:10 v/v) relative to NH_3 by using 1.0 M ^{15}N urea in DMSO at 25°C (Kaul et al., 2003)

[d] pK_a values of individual nitrogen atoms of neomycin determined using ^{1}H and ^{13}C HSQC NMR spectroscopy in D_2O at 25°C (Freire et al., 2007)

[e] This work

[f] The pK_a value of N-6' of neomycin C determined using ^{1}H NMR spectroscopy (in this work) is the average pK_a of the values of N-6' obtained using ^{1}H NMR spectroscopic data for 6'a (8.65) and 6'b (8.70), and the pK_a value of N-6''' of neomycin C determined using ^{1}H NMR spectroscopy (in this work) is the average pK_a of the values of N-6''' obtained using ^{1}H NMR spectroscopic data for 6'''a (8.72) and 6'''b (8.75)

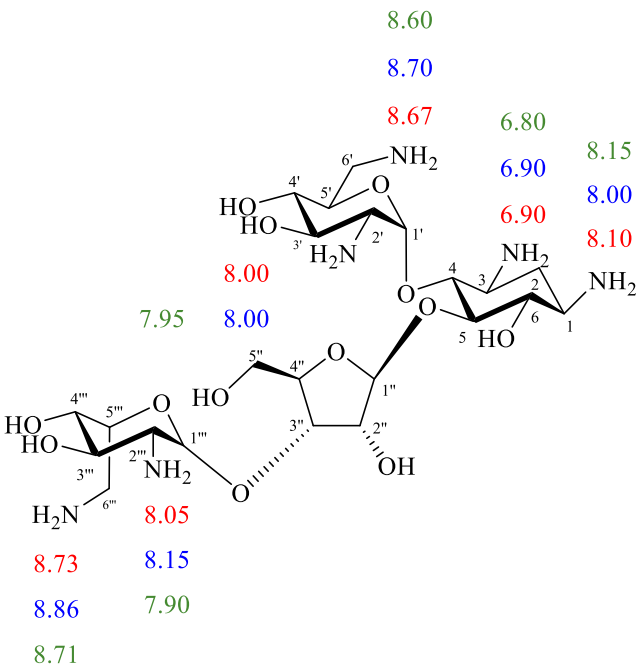


Fig. 4.30 pK_a values of individual nitrogen atoms of neomycin C determined using ^{1}H (red), ^{13}C (blue), and ^{15}N HMBC (green) NMR spectroscopy

After calculating the average pK_a values, using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopic data, of each individual nitrogen atom on neomycin C are: N-1 = 8.08, N-3 = 6.86, N-2' = 7.98, N-6' = 8.65, N-2''' = 8.03, and N-6''' = 8.76. The assignment order of the average ionisation constants is: N-6''' > N-6' > N-1 ≈ N-2' ≈ N-2''' > N-3 which is a revision of the assignment order reported in the literature. In the literature, the pK_a values of these amines on neomycin have been measured using one technique either ^{15}N NMR or ^{1}H-^{13}C HSQC NMR. Spectroscopy (Botto and Coxon, 1982; Kaul et al., 2003; Özen et al., 2006; Freire et al., 2007). However, in this study we have obtained the average pK_a values using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy of these amines on neomycin.

4.3.4. pK_a values of the individual amino groups of paromomycin (4)

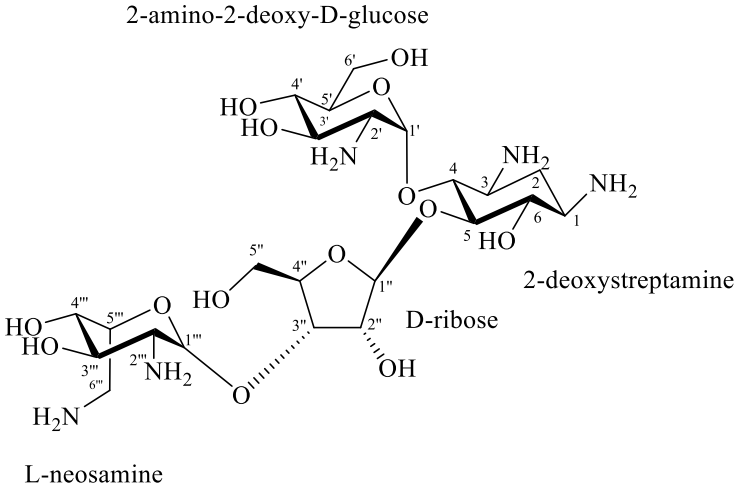


Fig. 4.31 Paromomycin (4)

Paromomycin has five primary amines. Those amines are substituents on two aminosugar rings: 2-amino-2-deoxy-D-glucose and L-neosamine and a central cyclohexane ring (2-deoxystreptamine). These three 6-membered rings are themselves *O*-substituents pendant from a D-ribose furanose ring. Paromomycin is a 4,5-*O*-disubstituted 2-deoxystreptamine (see Fig. 4.31). The chemical shifts of ^{1}H, ^{13}C, and ^{15}N HMBC of paromomycin at different pHs, shown in Tables 4.18, 4.19, and 4.20, were plotted against the pH values of the solution (all the tables are shown in the appendix). The nonlinear sigmoidal curves are shown in Figs. 4.32, 4.33, and 4.34. The pK_a values of individual nitrogen atoms of paromomycin, shown in Table 4.21 and Fig. 4.35, were extracted from the inflection points of the sigmoidal curves.

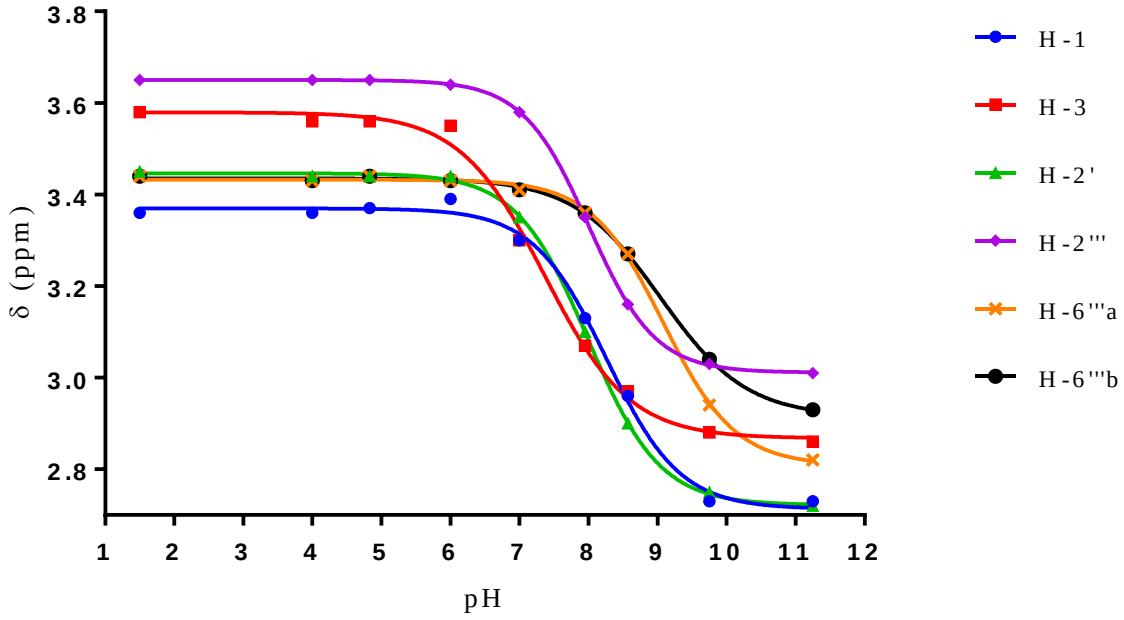


Fig. 4.32 NMR titration curves for the ^{1}H chemical shifts (δ) (500 MHz) of 0.335-0.208 M paromomycin were measured relative to TMSP in 99.97% D_2O at 25°C

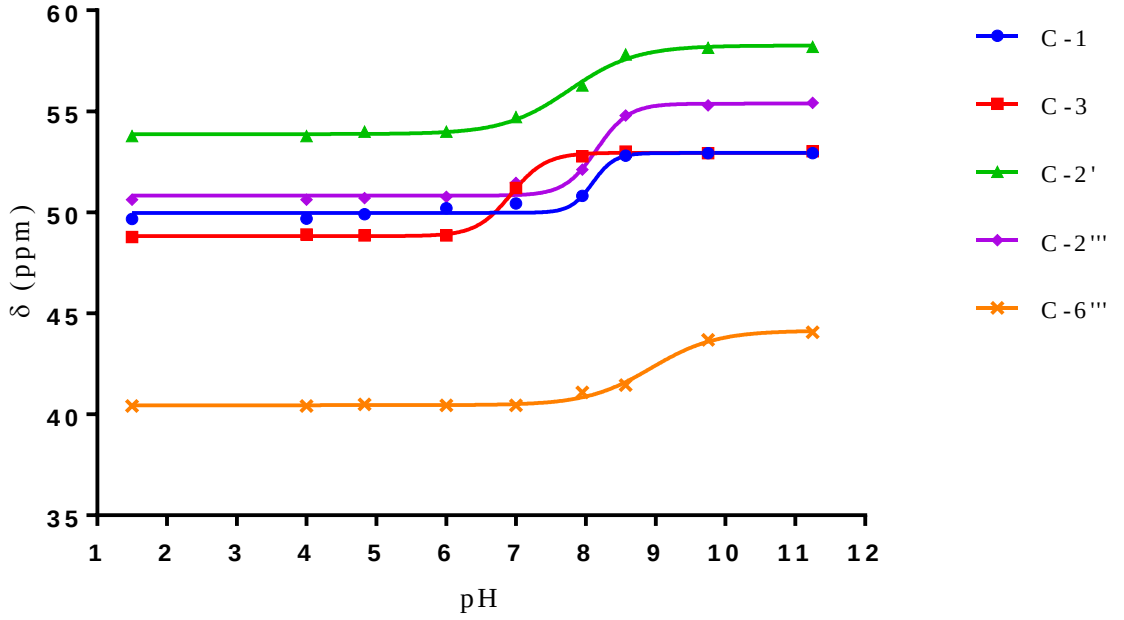


Fig. 4.33 NMR titration curves for the ^{13}C chemical shifts (δ) (125.77 MHz) of 0.335-0.208 M paromomycin were measured relative to TMSP in 99.97% D_2O at 25°C

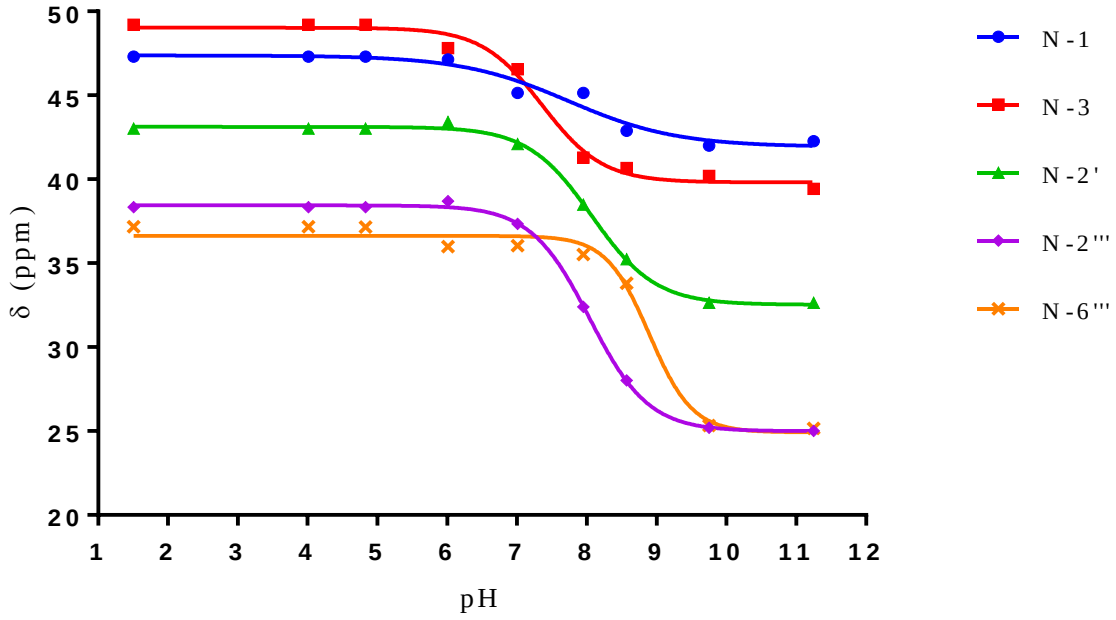


Fig. 4.34 NMR titration curves for the ^{15}N HMBC chemical shifts (δ) (50.67 MHz) of 0.335-0.208 M paromomycin were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C

Table 4.21 pK_a values of individual nitrogen atoms of paromomycin determined using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy in this work and then compared with the published data, as indicated

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'	N-2'''	N-6'''
^{15}N NMR [a]	8.20	6.50	8.07	7.91	9.13
^{15}N NMR [b]	8.65	7.07	8.33	8.25	9.46
^{1}H NMR [c]	8.25	6.45	8.10	8.20	9.15 [d]
^{13}C NMR [c]	8.10	6.55	8.00	8.11	9.00
^{15}N HMBC NMR [c]	8.00	6.50	8.10	8.00	9.10

[a] pK_a values of individual nitrogen atoms of paromomycin determined using ^{15}N NMR spectroscopy in H_2O/D_2O (85: 15 v/v) relative to NH_3 using 1 M [^{15}N] urea in DMSO at 25°C (Barbieri and Pilch, 2006)

[b] pK_a values of individual nitrogen atoms of paromomycin determined using ^{15}N NMR spectroscopy in H_2O/D_2O (85: 15 v/v) relative to NH_3 using 1 M [^{15}N] urea in DMSO at 25°C (Kaul et al., 2003)

[c] This work

[d] The pK_a value of N-6''' of paromomycin determined using ^{1}H NMR spectroscopy (in this work) is the average pK_a of the values of N-6''' obtained using ^{1}H NMR spectroscopic data for 6'''a (9.14) and 6'''b (9.16)

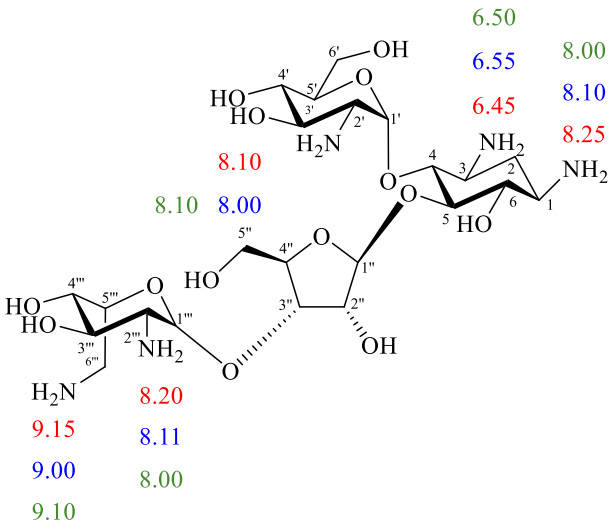


Fig. 4.35 pK_a values of individual nitrogen atoms of paromomycin determined using ^{1}H (red), ^{13}C (blue), and ^{15}N HMBC (green) NMR spectroscopy

After calculating the average pK_a values, using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopic data, for each individual nitrogen atom on paromomycin are: N-1 = 8.11, N-3 = 6.50, N-2' = 8.06, N-2''' = 8.10, and N-6''' = 9.08, the new assignment order of the average ionisation constants is: N-6''' > N-1 ≈ N-2' ≈ N-2''' > N-3. These pK_a values require a minor revision of the assignment order reported in the literature. In the literature, the pK_a values of these amines on paromomycin have been measured using one technique which is ^{15}N NMR. Spectroscopy (Kaul et al., 2003; Barbieri and Pilch, 2006). However, in this study we have obtained the average pK_a values using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy of these amines on paromomycin.

4.3.5. pK_a values of the individual amino groups of tobramycin (5)

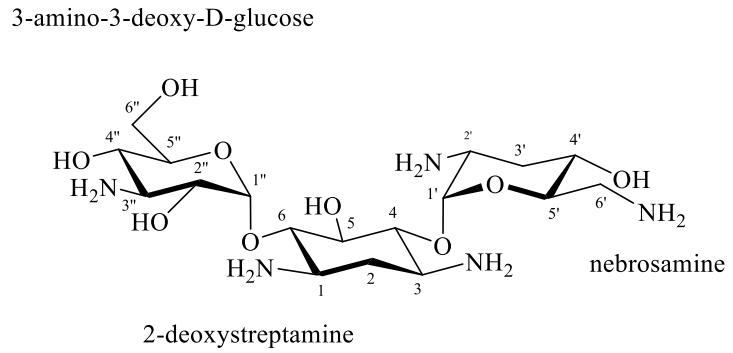


Fig. 4.36 Tobramycin (5)

Tobramycin has five primary amines. Those amines are substituents on two aminosugar rings: 3-deoxykanosamine (nebrosamine) and 3-amino-3-deoxy-D-glucose, and a central cyclohexane ring (2-deoxystreptamine). Tobramycin is a 4,6-*O*-disubstituted 2-deoxystreptamine (see Fig. 4.36). The pK_a determinations using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy were repeated twice. The average of the chemical shifts of ^{1}H, ^{13}C, and ^{15}N HMBC of tobramycin at different pHs, shown in Tables 4.22, 4.23, and 4.24, were plotted against the pH values of the solution (all the tables are shown in the appendix). The nonlinear sigmoidal curves are shown in Figs. 4.37, 4.38, and 4.39. The pK_a values of individual nitrogen atoms of tobramycin, shown in Table 4.25 and Fig. 4.40, were extracted from the inflection points of the sigmoidal curves.

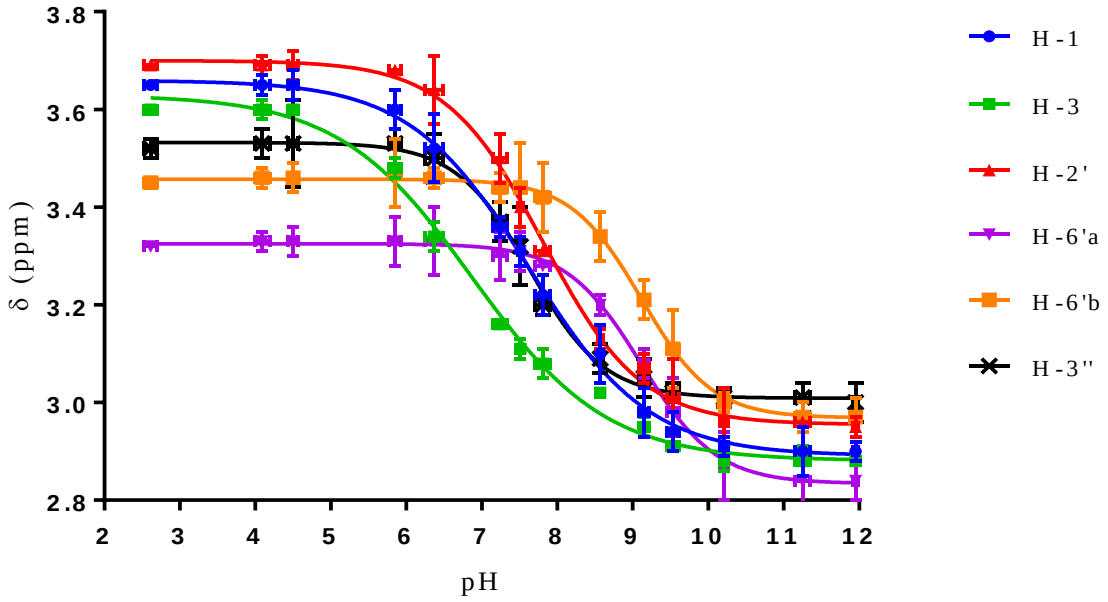


Fig. 4.37 NMR titration curves for the ^{1}H chemical shifts (δ) (500 MHz) of 0.251-0.132 M tobramycin were measured relative to TMSP in 99.97% D_2O at 25°C

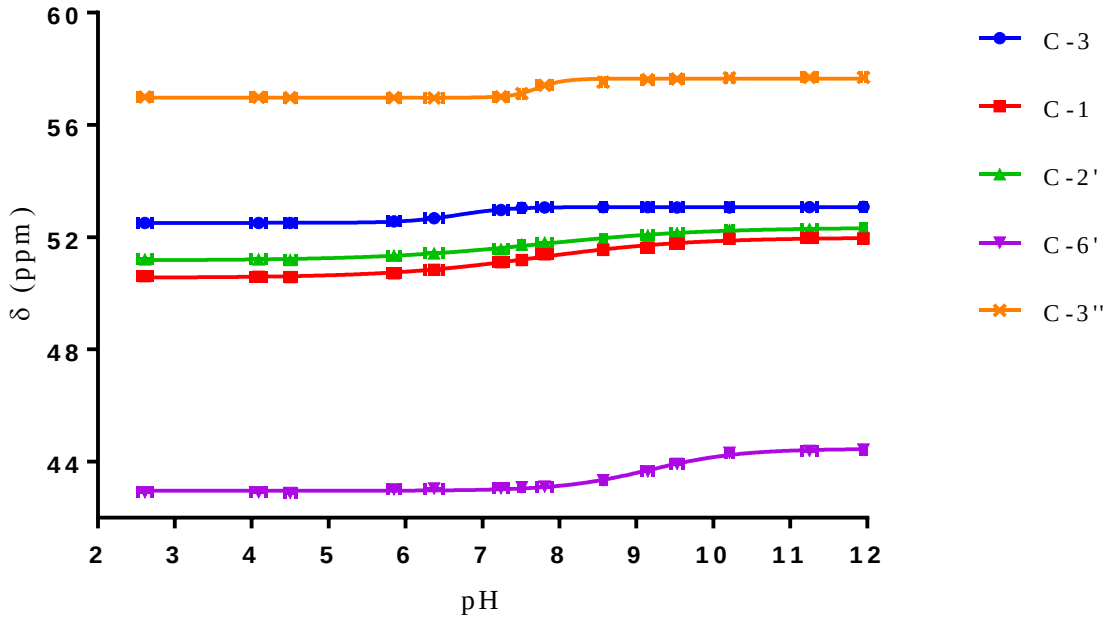


Fig. 4.38 NMR titration curves for the ^{13}C chemical shifts (δ) (125.77 MHz) of 0.251-0.132 M tobramycin were measured relative to TMSP in 99.97% D_2O at 25°C

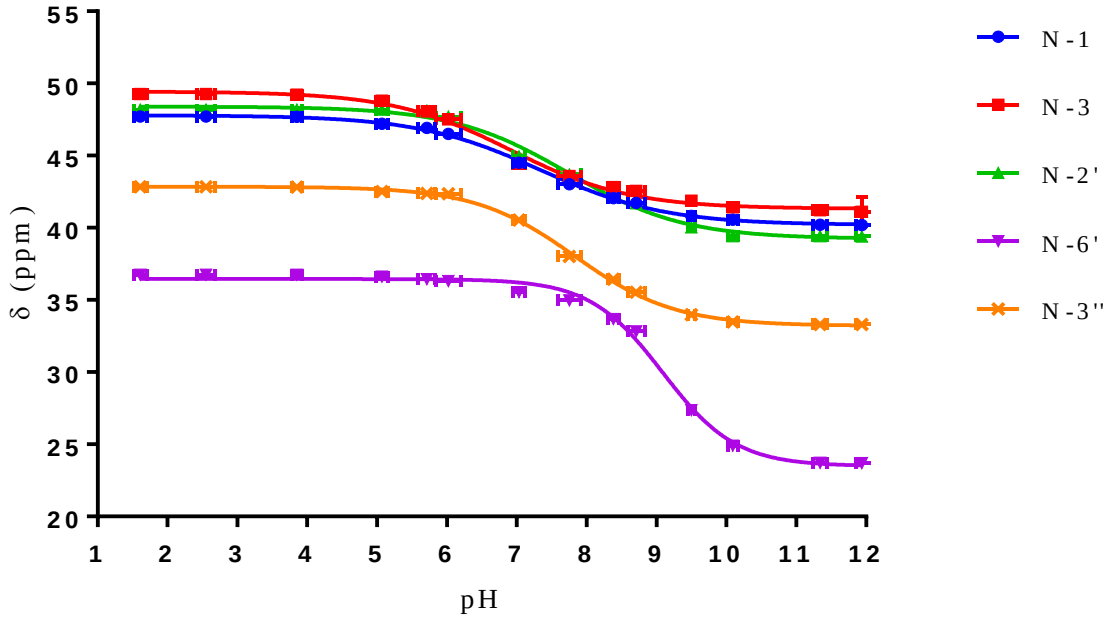


Fig. 4.39 NMR titration curves for the ^{15}N HMBC chemical shifts (δ) (50.67 MHz) of 0.740-0.370 M tobramycin were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C

Table 4.25 pK_a values of individual nitrogen atoms of tobramycin determined using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy in this work and then compared with the published data, as indicated

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'	N-6'	N-3''
^{15}N NMR [a]	7.40	6.20	7.60	8.60	7.40
^{1}H NMR [b]	7.30	6.60	7.50	8.40	7.30
^{15}N NMR [b]	7.40	6.40	7.70	8.50	7.40
^{1}H NMR [c]	7.51 ±0.03	6.60 ±0.05	7.80 ±0.05	9.07 ±0.10 [d]	7.62 ±0.08
^{13}C NMR [c]	7.61 ±0.07	6.80 ±0.15	7.71 ±0.07	9.15 ±0.05	7.71 ±0.03
^{15}N HMBC NMR [c]	7.55 ±0.05	6.70 ±0.05	7.75 ±0.05	9.10 ±0.05	7.70 ±0.05

[a] pK_a values of individual nitrogen atoms of tobramycin determined using ^{15}N NMR spectroscopy in H_2O/D_2O (90: 10 v/v) relative to $^{15}NH_4Cl$ at 25°C (Dorman et al., 1976)

[b] pK_a values of individual nitrogen atoms of tobramycin determined using ^{1}H NMR spectroscopy and ^{15}N NMR spectroscopy in D_2O relative to TMS at 25°C (Pagano et al., 2011)

[c] This work

[d] The pK_a value of N-6' of tobramycin determined using ^{1}H NMR spectroscopy (in this work) is the average pK_a of the values of N-6' obtained using ^{1}H NMR spectroscopic data for 6'a (9.05) and 6'b (9.10)

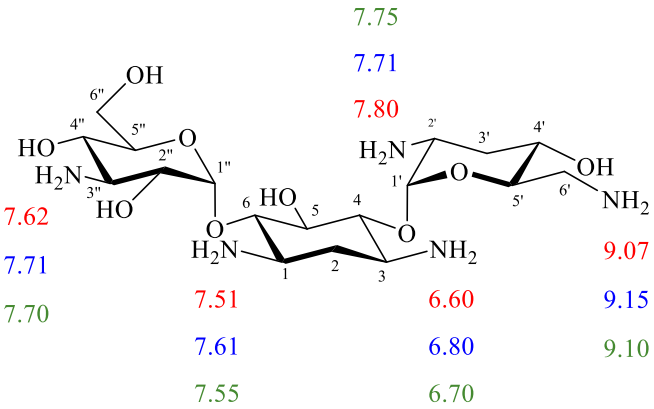


Fig. 4.40 pK_a values of individual nitrogen atoms of tobramycin determined using ^{1}H (red), ^{13}C (blue), and ^{15}N HMBC (green) NMR spectroscopy

After calculating the average pK_a values, using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopic data, of each individual nitrogen atom on tobramycin are: N-1 = 7.55, N-3 = 6.70, N-2' = 7.75, N-6' = 9.10, and N-3" = 7.68. The assignment order of the average ionisation constants within ± 0.05 is: N-6' > N-2' ≈ N-3" > N-1 > N-3. These pK_a values are consistent in assignment order with these reported in the literature (Dorman et al., 1976; Pagano et al., 2011).

4.3.6. pK_a values of the individual amino groups of kanamycin B (6)

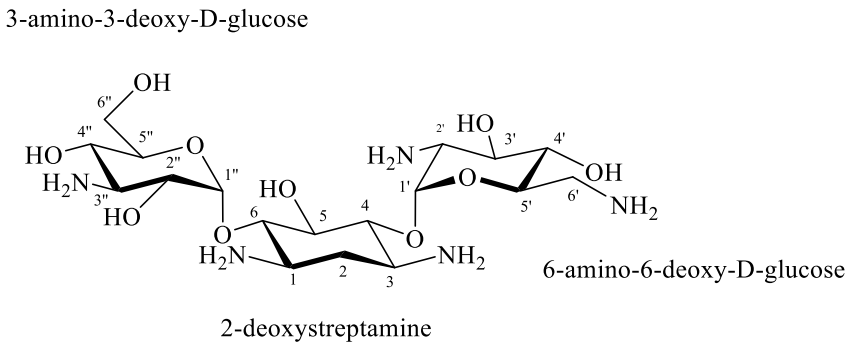


Fig. 4.41 Kanamycin B (6)

Kanamycin B has five amines. Those amines are substituents on two aminosugar rings: 3-amino-3-deoxy-D-glucose and 6-amino-6-deoxy-D-glucose, and a central cyclohexane ring (2-deoxystreptamine). Kanamycin B is a 4,6-*O*-disubstituted 2-deoxystreptamine (see Fig. 4.41). The chemical shifts of ^{1}H, ^{13}C, and ^{15}N HMBC of kanamycin B at different pHs, shown in Tables 4.26, 4.27, and 4.28, were plotted against the pH values of the solution (all the tables are shown in the appendix). The nonlinear sigmoidal are shown in Figs. 4.42, 4.43, and 4.44. The pK_a values of individual nitrogen atoms of kanamycin B, shown in Table 29 and Fig. 4.45, were extracted from the inflection points of the sigmoidal curves.

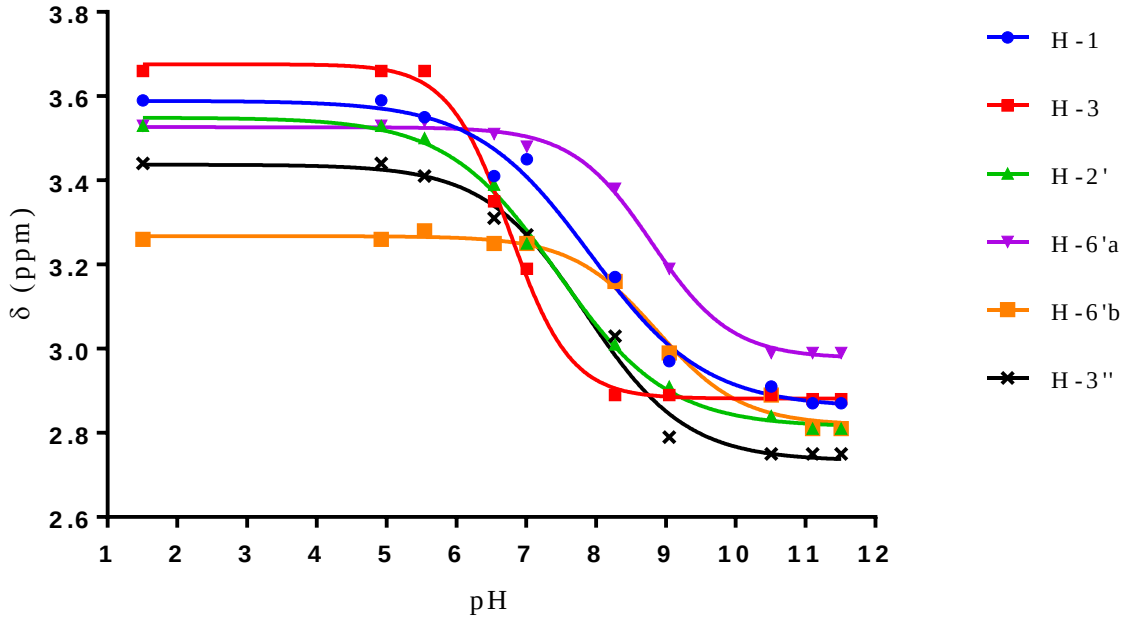


Fig. 4.42 NMR titration curves for the ^{1}H chemical shifts (δ) (500 MHz) of 1.315-0.822 M kanamycin B were measured relative to TMSP in 99.97% D_2O at 25°C

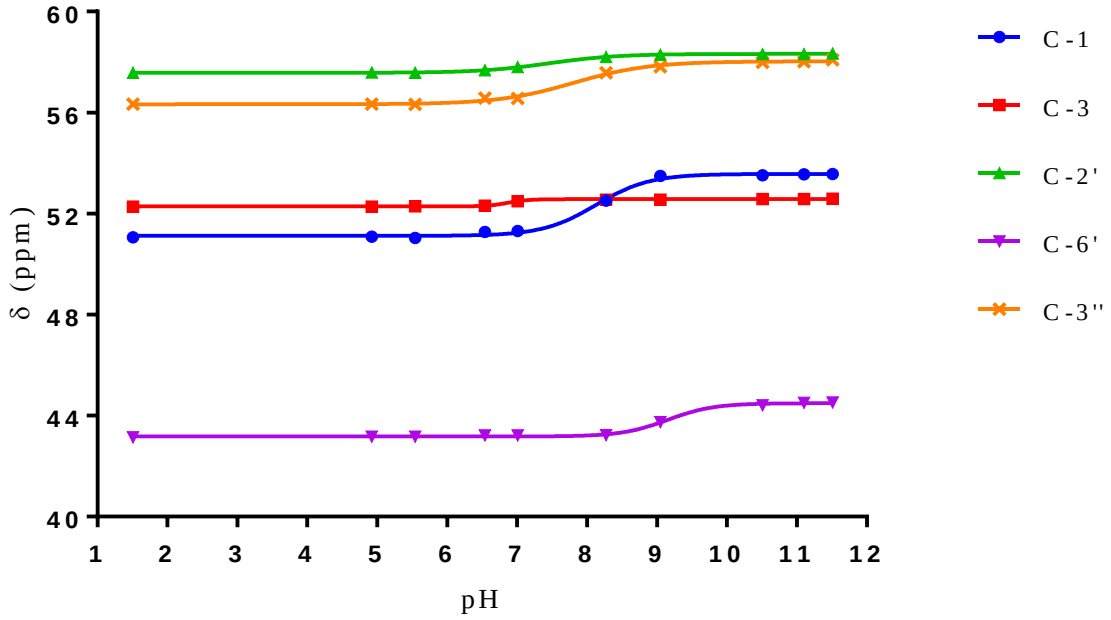


Fig. 4.43 NMR titration curves for the ^{13}C chemical shifts (δ) (125.77 MHz) of 1.315-0.822 M kanamycin B were measured relative to TMSP in 99.97% D_2O at 25°C

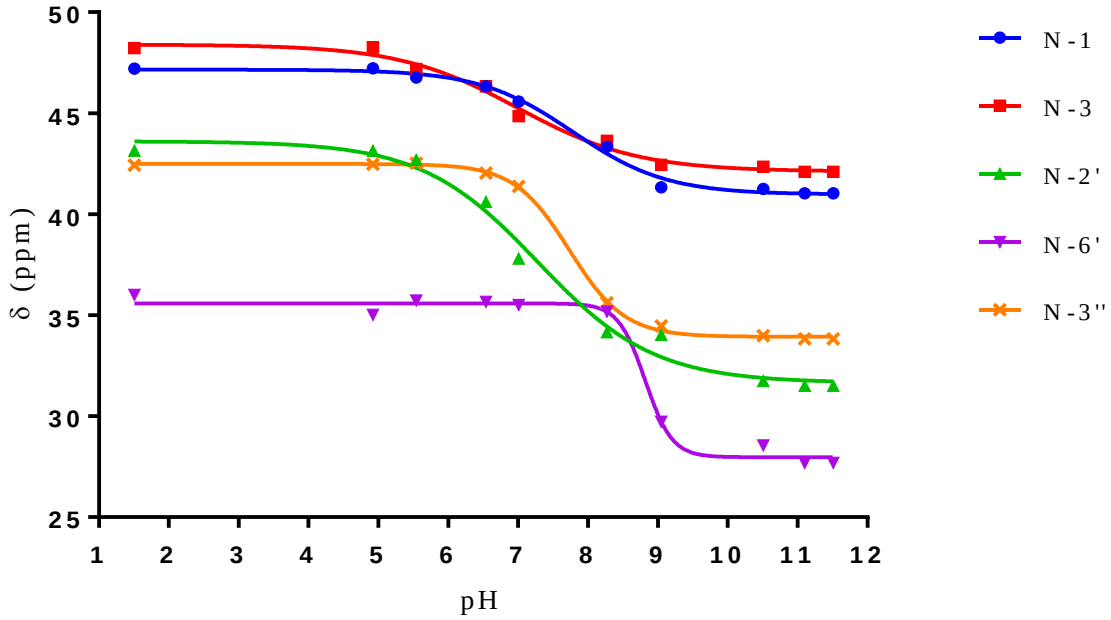


Fig. 4.44 NMR titration curves for the ^{15}N HMBC chemical shifts (δ) (50.67 MHz) of 1.315-0.822 M kanamycin B were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C

Table 4.29 pK_a values of individual nitrogen atoms of kanamycin B determined using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy in this work and then compared with the published data, as indicated

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'	N-6'	N-3''
^{1}H NMR [a]	8.12	6.04	-	9.03	7.46
^{1}H NMR [b]	8.16	6.71	7.35	8.93[c]	7.60
^{13}C NMR [b]	8.10	6.80	7.40	9.10	7.70
^{15}N HMBC NMR [b]	8.05	6.85	7.35	8.90	7.65

[a] pK_a values of individual nitrogen atoms of kanamycin A (note, which lacks an N-2' amine) determined using ^{1}H NMR spectroscopy in D_2O relative to TSP at 25°C (Gutiérrez-Moreno et al., 2012). Note also that there are no literature data for the pK_a values of kanamycin B.

[b] This work

[c] The pK_a value of N-6' of kanamycin B determined using ^{1}H NMR spectroscopy (in this work) is the average pK_a of the values of N-6' obtained using ^{1}H NMR spectroscopic data for 6'a (8.95) and 6'b (8.90)

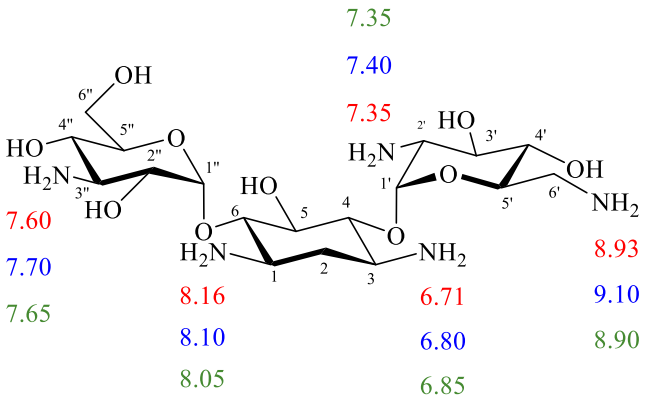


Fig. 4.45 pK_a values of individual nitrogen atoms of kanamycin B determined using ^{1}H (red), ^{13}C (blue), and ^{15}N HMBC (green) NMR spectroscopy

After calculating the average pK_a values, using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopic data, of each individual nitrogen atom on kanamycin B are: N-1 = 8.10, N-3 = 6.78, N-2' = 7.36, N-6' = 8.97, and N-3" = 7.65. The assignment order of the average ionisation constants is: N-6' > N-1 > N-3" > N-2' > N-3. In the absence of any kanamycin B published pK_a data determined using NMR spectroscopy, these are therefore reported for the first time.

4.3.7. pK_a values of the individual amino groups of netilmicin (7)

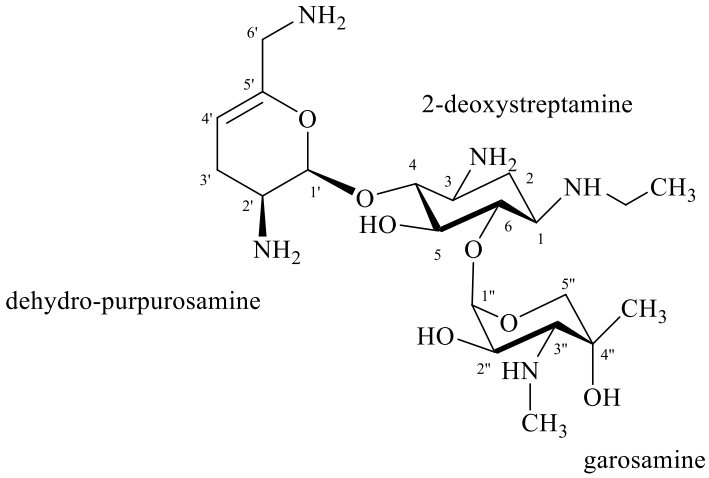


Fig. 4.46 Netilmicin (7)

Netilmicin includes five amines, which are substituents on two aminosugar rings: dehydro-purpurosamine and garosamine and a central cyclohexane ring (2-deoxystreptamine) (see Fig. 4.46). There are two different *N*-alkyl substituents: *N*-ethyl on N-1 and *N*-methyl on N-3". The chemical shifts of ^{1}H, ^{13}C, and ^{15}N HMBC of netilmicin at different pHs, shown in Tables 4.30, 4.31, and 4.32, were plotted against the pH values of the solution (all the tables are shown in the appendix). The nonlinear sigmoidal curves are shown in Figs. 4.47, 4.48, and 4.49. The pK_a values of individual nitrogen atoms of netilmicin, shown in Table 4.33 and Fig. 4.50, were extracted from the inflection points of the sigmoidal curves.

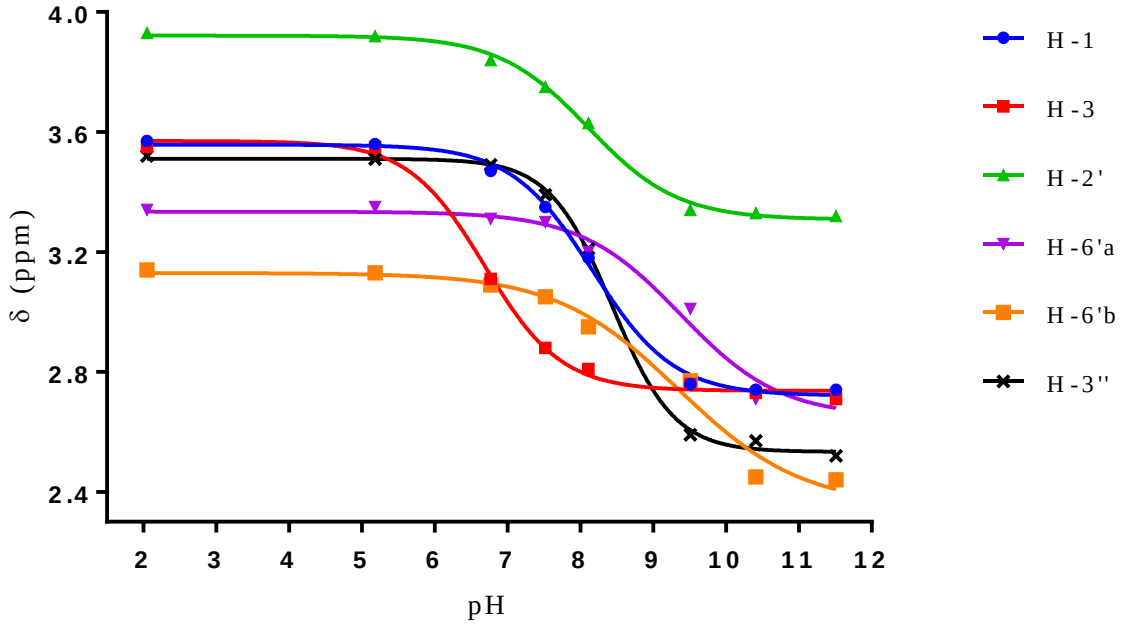


Fig. 4.47 NMR titration curves for the ^{1}H chemical shifts (δ) (500 MHz) 0.506-0.434 M netilmicin were measured relative to TMSP in 99.97% D_2O at 25°C

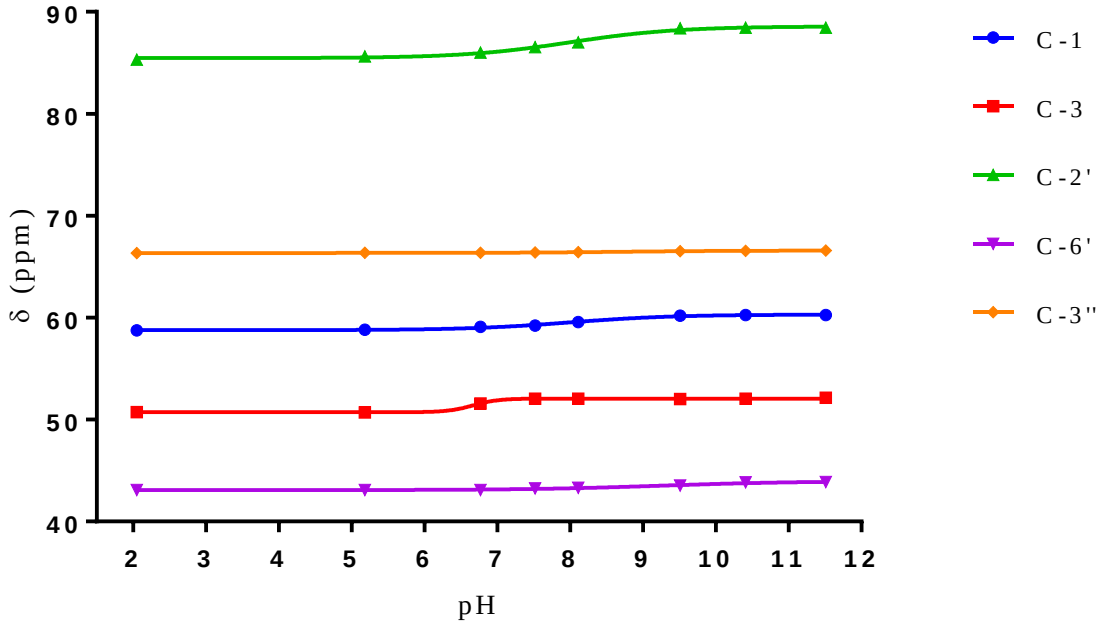


Fig. 4.48 NMR titration curves for the ^{13}C chemical shifts (ppm) (125.77 MHz) of 0.506-0.434 M netilmicin were measured relative to TMSP in 99.97% D_2O at 25°C

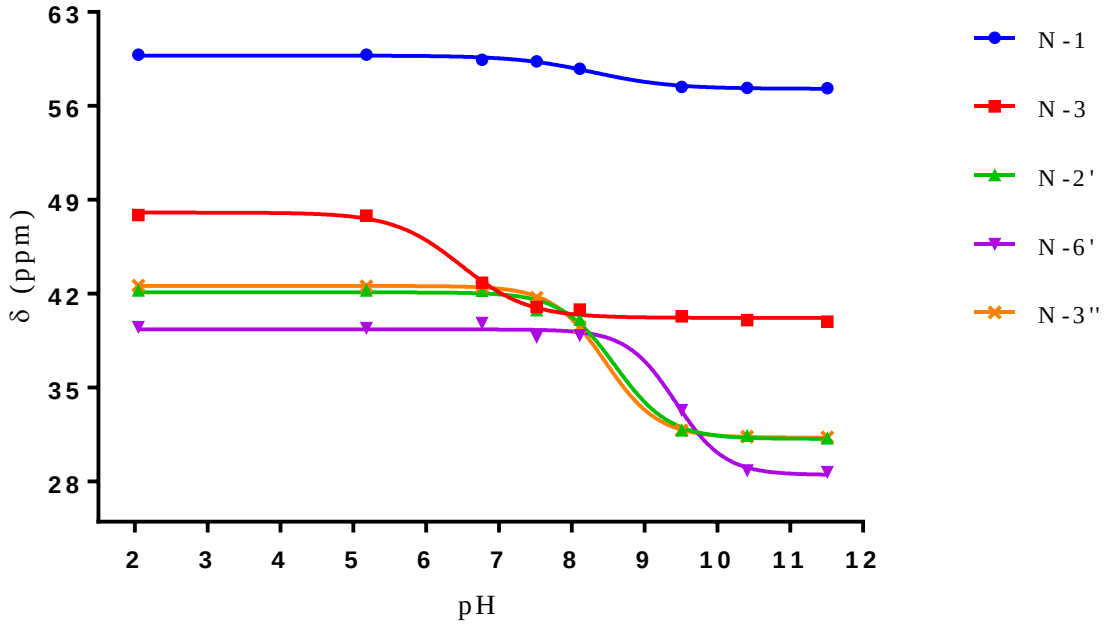


Fig. 4.49 NMR titration curves for the ^{15}N HMBC chemical shifts (δ) (50.67 MHz) of 0.506-0.434 M netilmicin were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C

Table 4.33 pK_a values of individual nitrogen atoms of netilmicin determined using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy in this work

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'	N-6'	N-3''
^{1}H NMR [a]	8.15	6.55	8.10	9.27 [b]	8.48
^{13}C NMR [a]	8.11	6.50	8.11	9.32	8.50
^{15}N HMBC NMR [a]	8.20	6.51	8.23	9.37	8.45

[a] This work. As far as can be determined, there are no literature data for the pK_a values of netilmicin.

[b] The pK_a value of N-6' of netilmicin determined using ^{1}H NMR spectroscopy (in this work) is the average pK_a of the values of N-6' obtained using ^{1}H NMR spectroscopic data for 6'a (9.29) and 6'b (9.25)

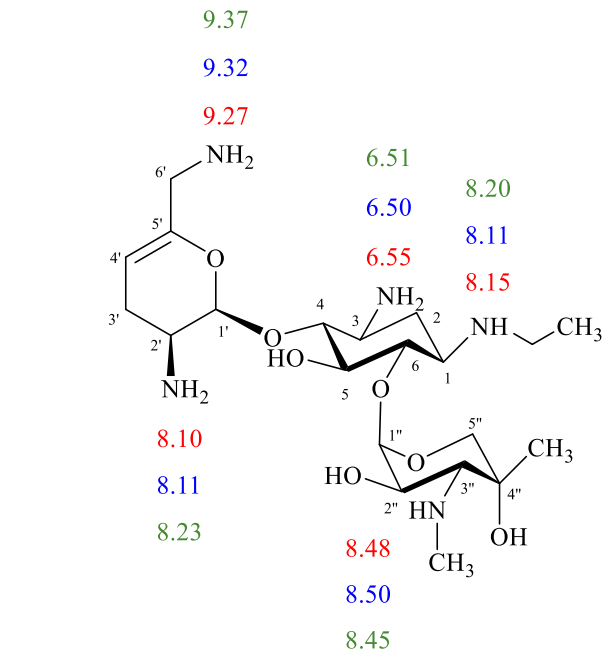


Fig. 4.50 pK_a values of individual nitrogen atoms of netilmicin determined using ^{1}H (red), ^{13}C (blue), and ^{15}N HMBC (green) NMR spectroscopy

In the absence of any netilmicin published pK_a data, these are therefore reported for the first time. The average pK_a values are: N-1 = 8.15, N-3 = 6.52, N-2' = 8.14, N-6' = 9.29, and N-3" = 8.47 and these are assigned in this order: N-6' > N-3" > N-1 ≈ N-2' > N-3.

4.3.8. pK_a values of the individual amino groups of sisomicin (8)

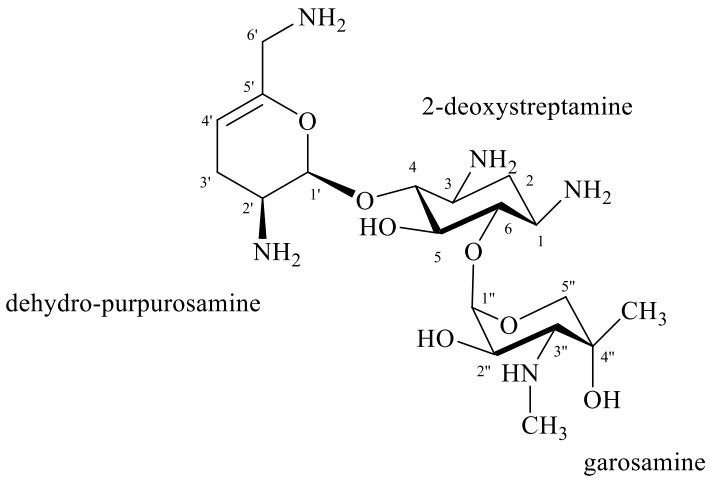


Fig. 4.51 Sisomicin (8)

Sisomicin includes four primary amines and a secondary (*N*-methyl) amine. Those amines are substituents on two aminosugar rings: dehydro-purpurosamine and garosamine and a central cyclohexane ring (2-deoxystreptamine) (see Fig. 4.51). The chemical structure of sisomicin is similar to that of netilmicin, with a primary amine as N-1 for the *N*-ethyl of netilmicin the only difference (see Figs. 4.1, 4.46 and 4.51). The chemical shifts of ^{1}H, ^{13}C, and ^{15}N HMBC of sisomicin at different pHs, shown in Tables 4.34, 4.35, and 4.36, were plotted against the pH values of the solution (all the tables are shown in the appendix). The nonlinear sigmoidal curves are shown in Figs. 4.52, 4.53, and 4.54. The pK_a values of individual nitrogen atoms of sisomicin, shown in Table 4.37 and Fig. 4.55, were extracted from the inflection points of the sigmoidal curves.

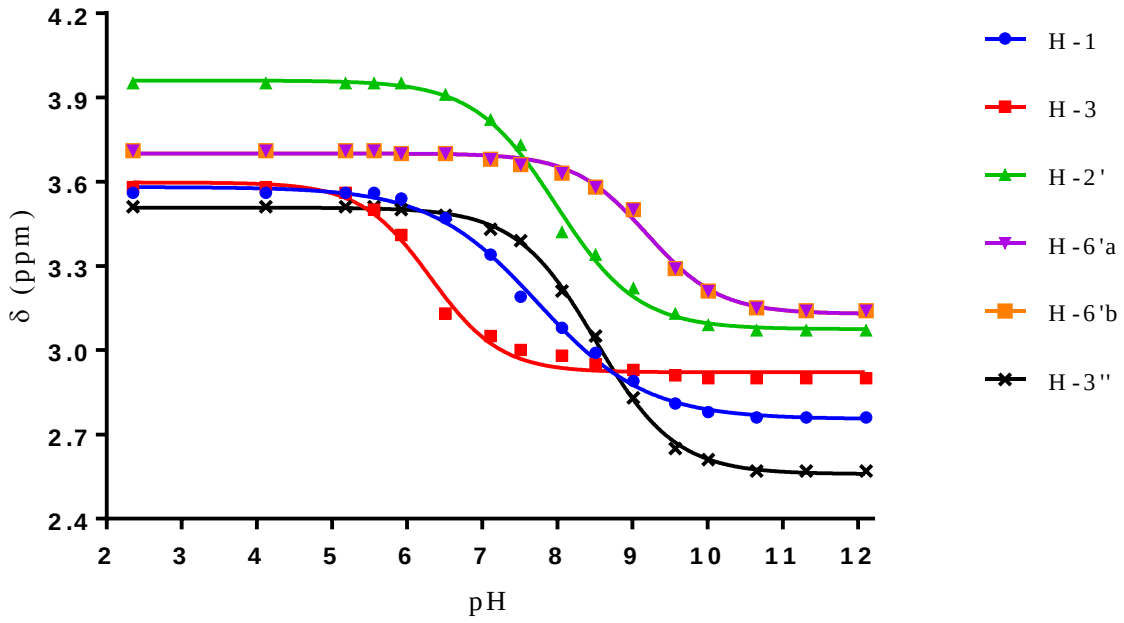


Fig. 4.52 NMR titration curves for the ^{1}H chemical shifts (δ) (500 MHz) of 0.083-0.063 M sisomicin were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

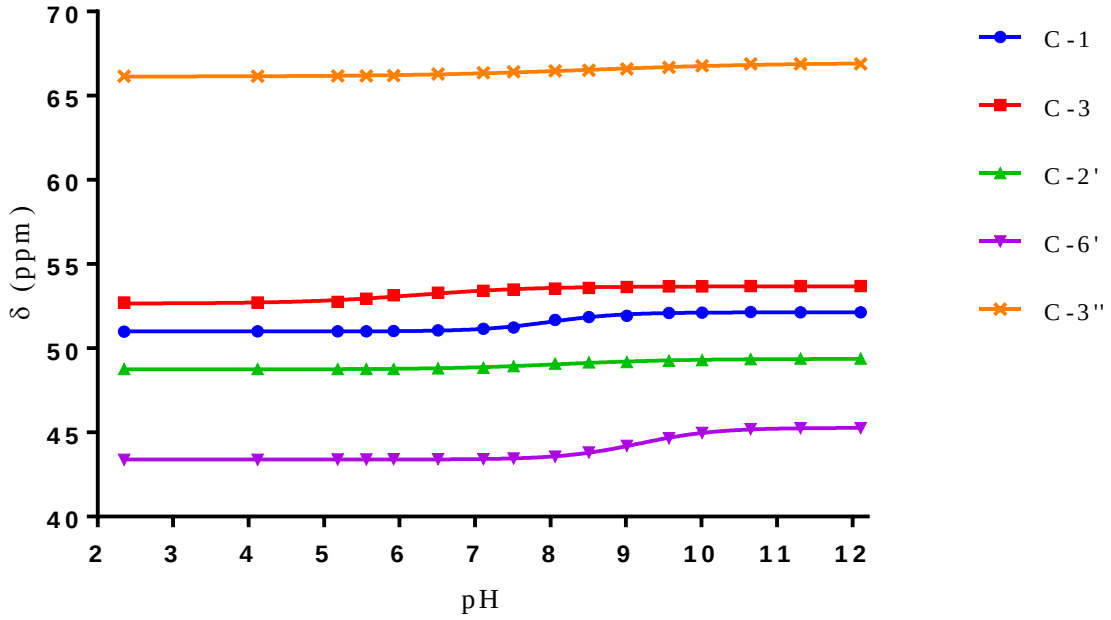


Fig. 4.53 NMR titration curves for the ^{13}C chemical shifts (δ) (125.77 MHz) of 0.083-0.063 M sisomicin were measured relative to TMSP in 99.97% D_2O at 25°C

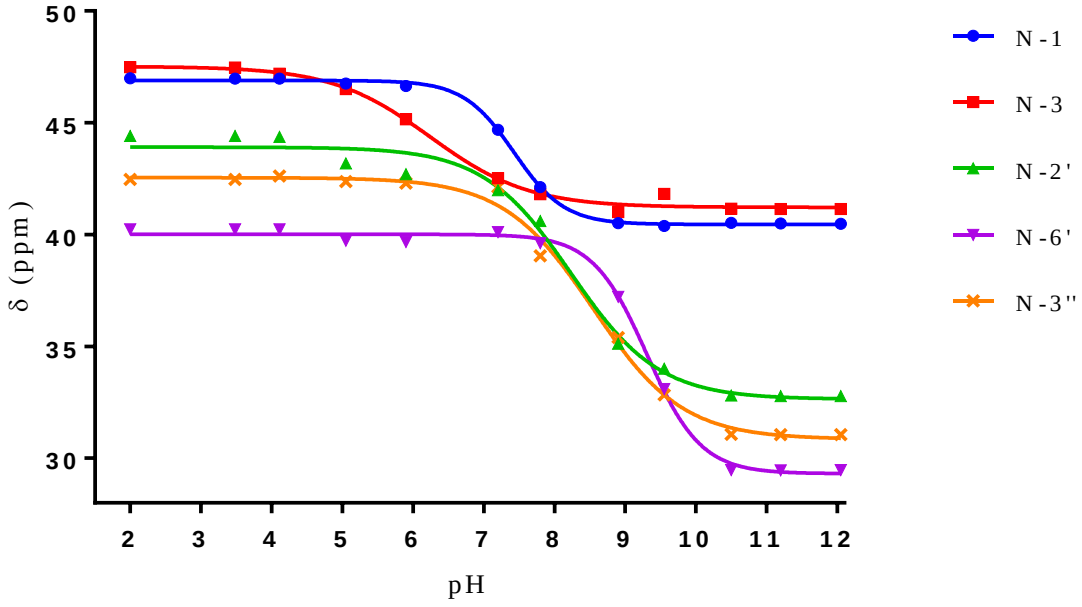


Fig. 4.54 NMR titration curves for the ^{15}N HMBC chemical shifts (δ) (50.67 MHz) of 0.160-0.112 M sisomicin were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C

Table 4.37 pK_a values of individual nitrogen atoms of sisomicin determined using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy in this work and then compared with the published data, as indicated

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'	N-6'	N-3''
^{1}H NMR [a]	7.34	6.11	7.93	9.45	8.63
^{1}H NMR [b]	7.42	6.18	7.98	9.35 [c]	8.51
^{13}C NMR [b]	7.45	6.25	7.99	9.28	8.45
^{15}N HMBC NMR [b]	7.41	6.24	8.05	9.29	8.55

[a] pK_a values of individual nitrogen atoms of sisomicin determined using ^{1}H NMR spectroscopy in D_2O relative to TSP at 25°C (Krężel et al., 2004)

[b] This work

[c] The pK_a value of N-6' of sisomicin determined using ^{1}H NMR spectroscopy (in this work) is the average pK_a of the values of N-6' obtained using ^{1}H NMR spectroscopic data for 6'a (9.35) and 6'b (9.35), which gave the same value

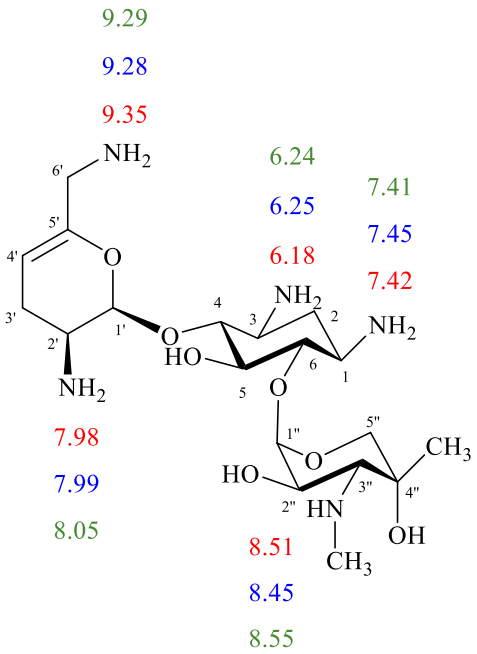


Fig. 4.55 pK_a values of individual nitrogen atoms of sisomicin determined using ^{1}H (red), ^{13}C (blue), and ^{15}N HMBC (green) NMR spectroscopy

After calculating the average pK_a values, using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopic data, of each individual nitrogen atom on sisomicin are: N-1 = 7.42, N-3 = 6.22, N-2' = 8.00, N-6' = 9.30, and N-3'' = 8.50. The assignment order of the average ionisation constants is: N-6' > N-3'' > N-2' > N-1 > N-3. These pK_a values are consistent in magnitude and in assignment order with these reported in the literature (Krężel et al., 2004).

4.3.9. pK_a values of the individual amino groups of amikacin (9)

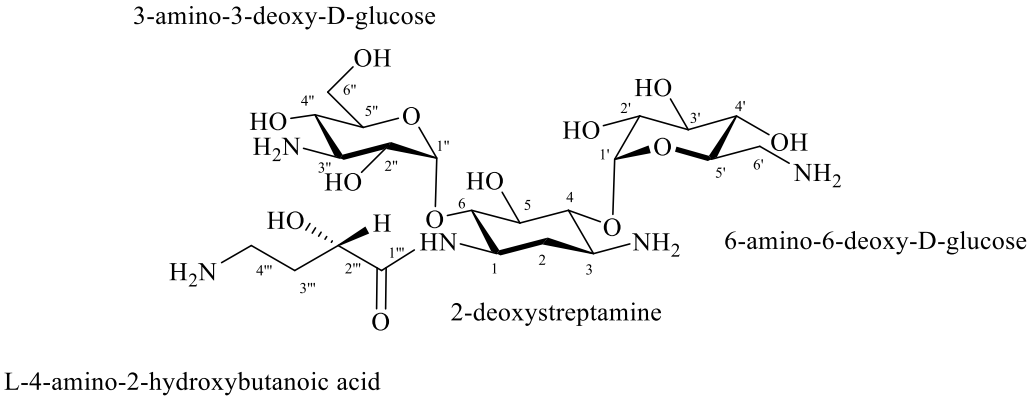


Fig. 4.56 Amikacin (9)

Amikacin includes four amines, which are substituents on two aminosugar rings: 3-amino-3-deoxy-D-glucose and 6-amino-6-deoxy-D-glucose, a central cyclohexane ring (2-deoxystreptamine), and L-amino-α-hydroxybutanoic acid (see Fig. 4.56). The chemical shifts of ^{1}H, ^{13}C, and ^{15}N HMBC of amikacin at different pHs, shown in Tables 4.38, 4.39, and 4.40, were plotted against the pH values of the solution (all the tables are shown in the appendix). The nonlinear sigmoidal curves are shown in Fig. 4.57, 4.58, and 4.59. The pK_a values of the individual amino groups of amikacin, shown in Table 4.41 and Fig. 4.60, were extracted from the inflection points of the sigmoidal curves.

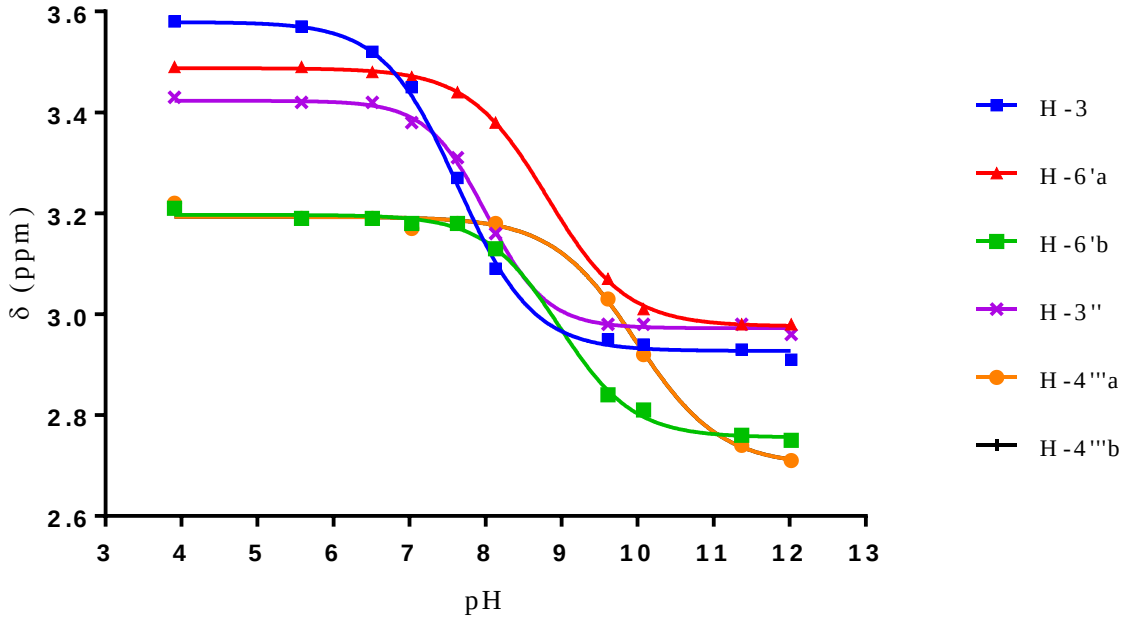


Fig. 4.57 NMR titration curves for the ^{1}H chemical shifts (δ) (500 MHz) of 0.896-0.597 M amikacin were measured relative to TMSP in 99.97% D_2O at 25°C

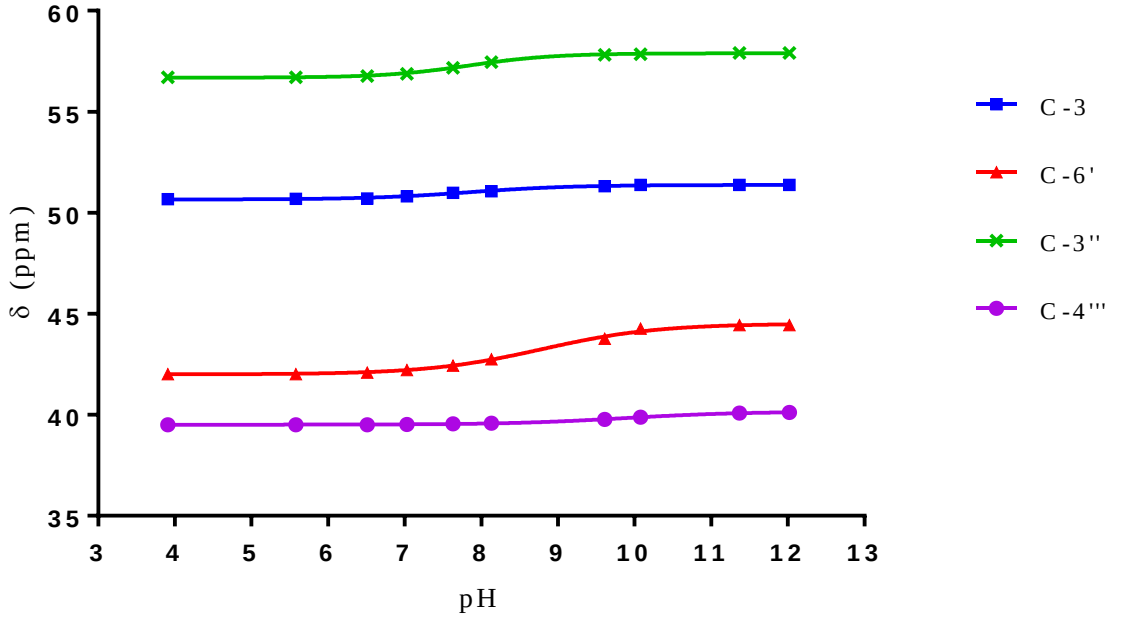


Fig. 4.58 NMR titration curves for the ^{13}C chemical shifts (δ) (125.77 MHz) of 0.896-0.597 M amikacin were measured relative to TMSP in 99.97% D_2O at 25°C

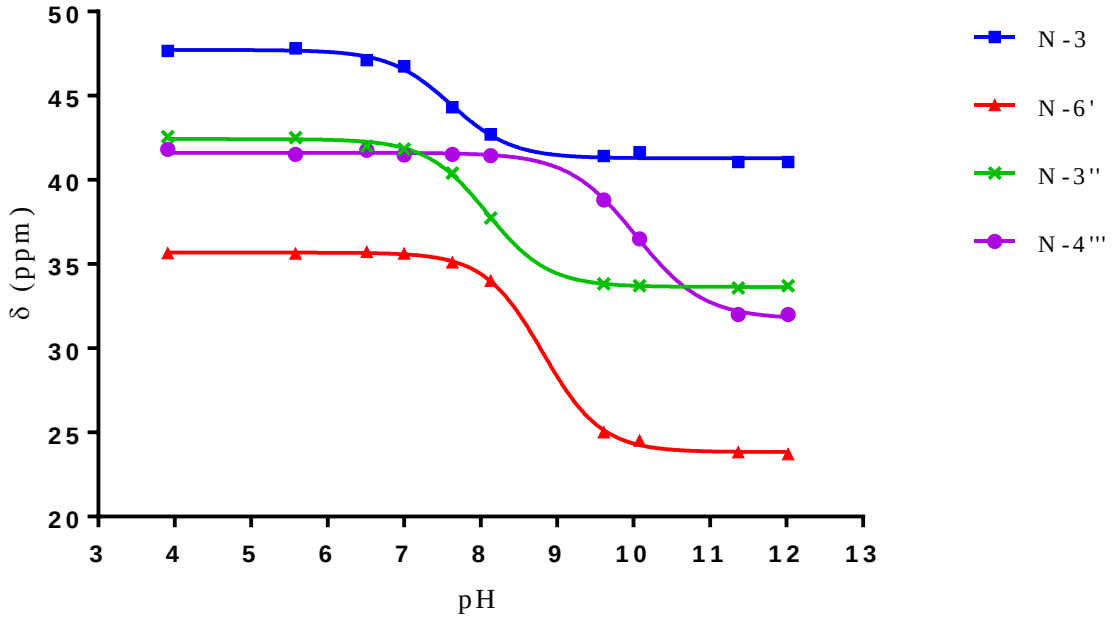


Fig. 4.59 NMR titration curves for the ^{15}N HMBC chemical shifts (δ) (50.67 MHz) of 0.896-0.597 M amikacin were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C

Table 4.41 pK_a values of individual nitrogen atoms of amikacin determined using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy in this work and then compared with the published data, as indicated

Individual nitrogen atoms pK_a / Method	N-3	N-6'	N-3''	N-4'''
^{15}N NMR [a]	7.62	8.92	8.13	9.70
^{1}H NMR [b]	7.70	8.93 [c]	8.05	9.92
^{13}C NMR [b]	7.63	8.70	8.00	9.85
^{15}N HMBC NMR [b]	7.60	8.80	8.10	9.90

[a] pK_a values of individual nitrogen atoms of amikacin determined using ^{15}N NMR spectroscopy in H_2O/D_2O (85: 15 v/v) relative to $^{15}NH_4Cl$ at 25°C (Cox and Serpersu, 1997)

[b] This work

[c] The pK_a value of N-6' of amikacin determined using ^{1}H NMR spectroscopy (in this work) is the average pK_a of the values of N-6' obtained using ^{1}H NMR spectroscopic data for 6'a (8.90) and 6'b (8.96)

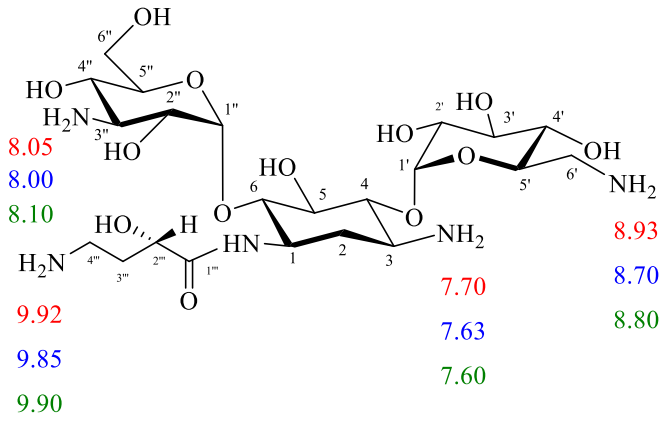


Fig. 4.60 pK_a values of individual nitrogen atoms of amikacin determined using ^{1}H (red), ^{13}C (blue), and ^{15}N HMBC (green) NMR spectroscopy

After calculating the average pK_a values, using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopic data, of each amino group on amikacin are: N-3 = 7.64, N-6' = 8.81, N-3" = 8.05, and N-4"' = 9.89. The assignment order of the average ionisation constants is: N-4"' > N-6' > N-3" > N-3. These pK_a values are consistent in magnitude and in assignment order with these reported in the literature (Cox and Serpersu, 1997).

4.3.10. pK_a values of the individual amino groups of streptomycin (10)

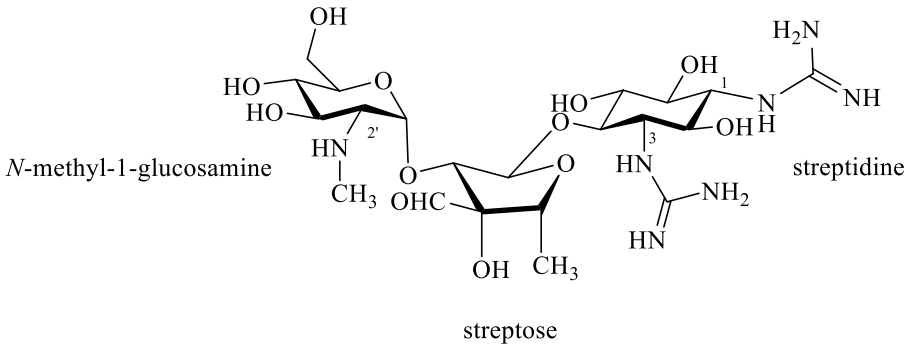


Fig. 4.61 Streptomycin

Streptomycin has seven nitrogen atoms distributed within two guanidine groups on streptidine and a secondary amine (*N*-methyl) on an *N*-methyl-1-glucosamine ring (see Fig. 4.61). The streptose ring with its 6-carbon atoms is a furanose, not a pyranose, and also it is not a linear sugar with its methyl and an aldehyde functional group. The chemical shifts of ^{1}H, ^{13}C, and ^{15}N HMBC of streptomycin at different pHs, shown in Tables 4.42, 4.43, and 4.44, were plotted against the pH values of the solution (all the tables are shown in the appendix). The nonlinear sigmoidal curves are shown in Figs. 4.62, 4.63, 4.64, and 4.65. The individual pK_a values of the two guanidine groups (N-1 and N-3) and the secondary amine (*N*-methyl) of streptomycin, shown in Table 4.45 and Fig. 4.66, were extracted from the inflection points of the sigmoidal curves.

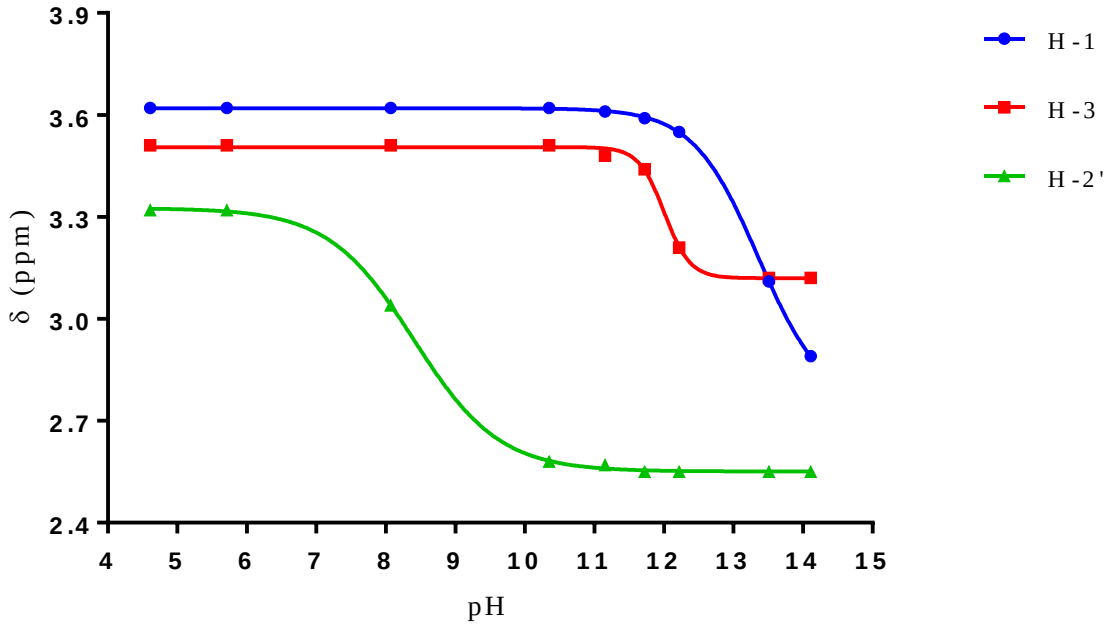


Fig. 4.62 NMR titration curves for the ^{1}H chemical shifts (δ) (500 MHz) of 0.738-0.527 M streptomycin were measured relative to TMSP in 99.97% D_2O at 25°C

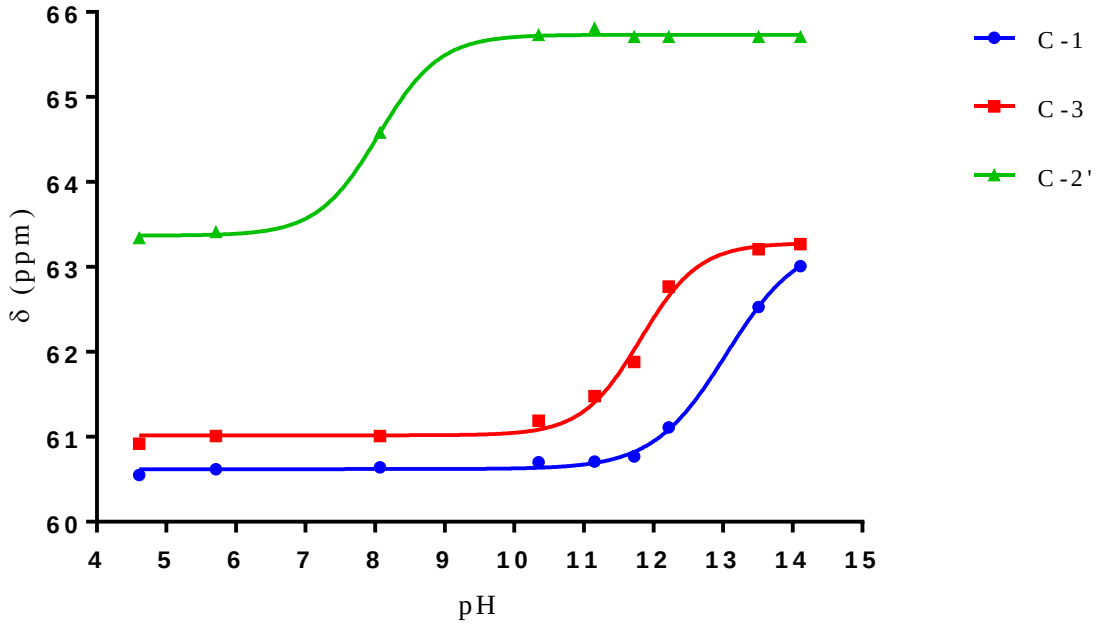


Fig. 4.63 NMR titration curves for the ^{13}C chemical shifts (δ) (125.77 MHz) of 0.738-0.527 M streptomycin were measured relative to TMSP in 99.97% D_2O at 25°C

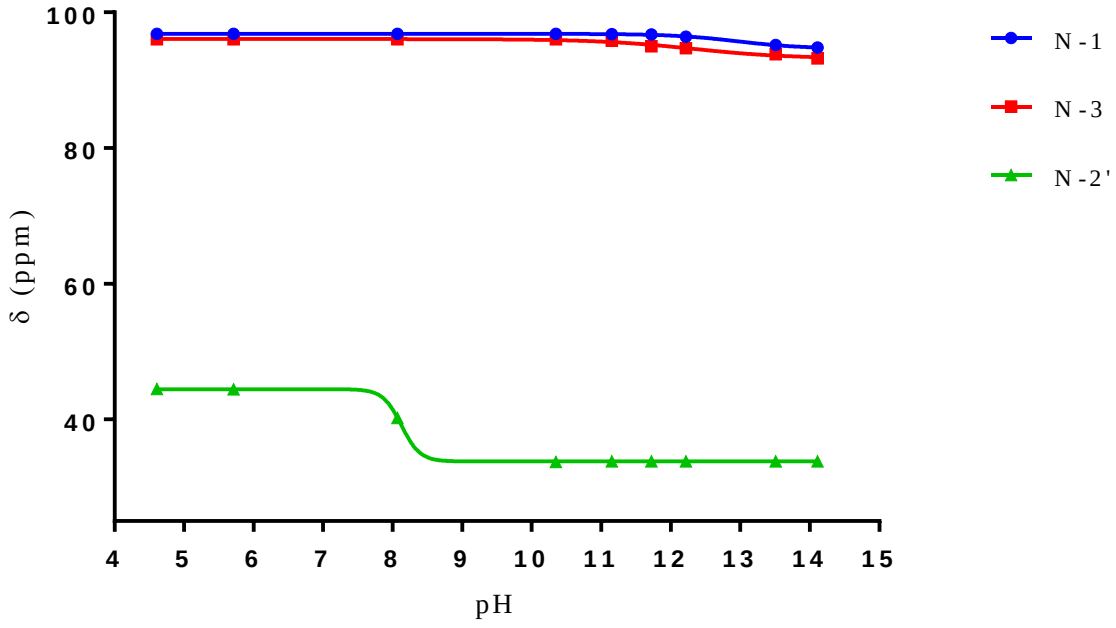


Fig. 4.64 NMR titration curves for the ^{15}N HMBC chemical shifts (δ) (50.67 MHz) of 0.738-0.527 M streptomycin were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C

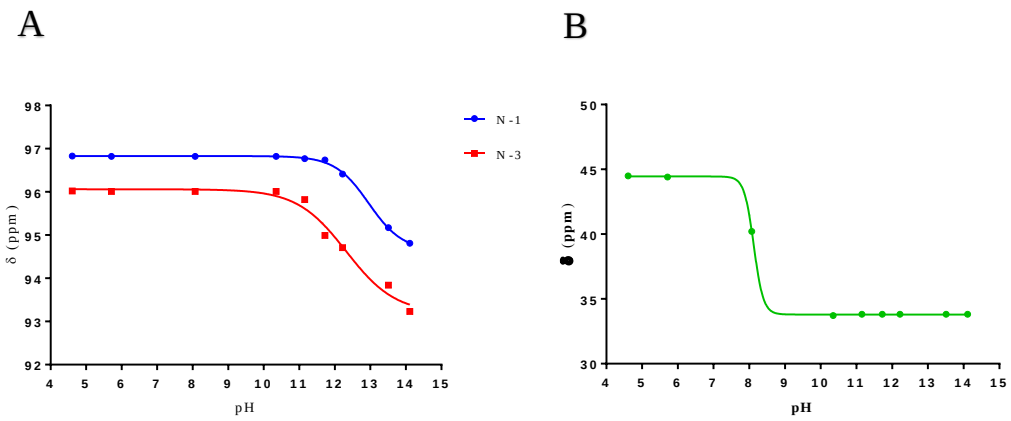


Fig. 4.65 NMR titration curves of A) N-1, N-3 and B) N-2', expanded from Fig. 4.55

Table 4.45 pK_a values of the two guanidine groups (N-1 and N-3) and the secondary amine (*N*-methyl) N-2' of streptomycin determined using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy in this work and then compared with the published data, as indicated

Individual nitrogen atoms pK_a / Method	N-1	N-3	N-2'
^{1}H NMR [a]	13.55	12.33	8.29
^{1}H NMR [b]	13.20	12.10	8.38
^{13}C NMR [b]	13.02	11.95	8.11
^{15}N NMR [b]	12.97	12.15	8.01

[a] pK_a values of individual nitrogen atoms of streptomycin determined using ^{1}H NMR spectroscopy in H_2O/D_2O (95: 05 v/v) relative to DSS at 25°C (Orgován and Noszál, 2012).

[b] This work

After calculating the average pK_a values, using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopic data, of the two guanidine groups (N-1 and N-3) and the secondary amine (*N*-methyl) on streptomycin are: N-1 (guanidine) = 13.06, N-3 (guanidine) = 12.06, and N-2' = 8.16. The assignment order of the average ionisation constants is: N-1 > N-3 > N-2'. These pK_a values are consistent in assignment order with these reported in the literature (Orgován and Noszál, 2012).

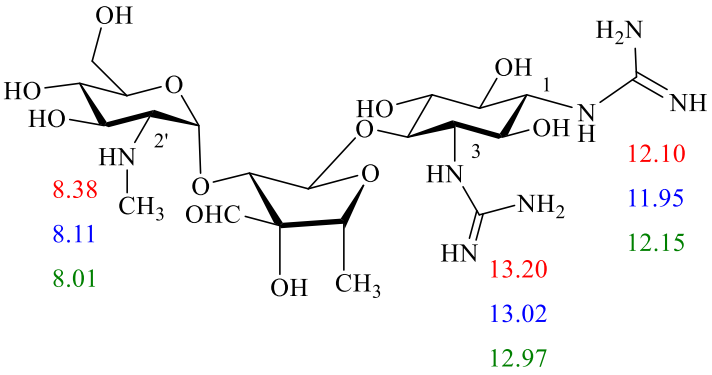


Fig. 4.66 pK_a values of individual nitrogen atoms of streptomycin determined using ^{1}H (red), ^{13}C (blue), and ^{15}N HMBC (green) NMR spectroscopy

4.3.11. General discussion of section 4.3

The pK_a values of N-3 of neamine (2) (6.50), paromomycin (4) (6.50), tobramycin (5) (6.70), kanamycin B (6) (6.78), netilmicin (7) (6.52), sisomicin (8) (6.22), and neomycin C (3) (6.86), were the lowest pK_a value among other amines on these aminoglycosides. The reason for the lowering of the pK_a values of N-3 amino groups compare to other amines located on these natural products is the inductive effect of the neighbouring NH_3^+-1 (short distance between N-1 and N-3 at 2-deoxystreptamine ring) (Cox and Serpersu, 1997; Krężel et al., 2004).

Depending on both the chemical structures and the acid-base properties of aminoglycosides, the amino substituents can be classified into four groups, as follows: primary amines, attached directly to the amino-sugar ring (R-NH_2), primary amino methylene groups (R-CH_2NH_2), primary amines in the aliphatic chains and the secondary amines attached directly to the amino-sugar ring. ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopic data indicated that the lowest pK_a values were the primary amines (R-NH_2) attached directly to the sugar ring.

Side-chain amino groups (on amikacin) and guanidine groups (on streptomycin) had the highest pK_a value. The average pK_a values for the primary amines attached directly to the aminosugar ring (R-NH_2) using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopic data is 7.53, for

the secondary amines is 8.32, for the primary amino methylene groups ($R\text{-}CH_2NH_2$) is 8.91, for the side-chain amino groups is 9.89, and for the guanidine groups is 12.56 (see Table 4.46). This observation might result from the side chain amino groups being less sterically hindered than the primary aminomethylene groups ($R\text{-}CH_2NH_2$) and the primary amines attached directly to the aminosugar rings ($R\text{-}NH_2$), respectively and the electron donating effects of alkyl groups on the pK_a values of the Side-chain amino groups and primary amino methylene groups ($R\text{-}CH_2NH_2$) (Krężel et al., 2004).

The pK_a values of the amino groups of netilmicin (7) and sisomicin (8) antibiotics are higher pK_a values (more basic) than the other aminoglycosides that contains 2-deoxystreptamine ring (see sections 4.1.7 for netilmicin and 4.1.8 for sisomicin). Some differences in their structures, methylation of N-3" and ethylation of N-1 in netilmicin (7) or methylation of N-3" in sisomicin (8). Moreover, the electron donationg effects of alkyl groups on N-1 and N-3" of netilmicin and on N-3" of sisomicin could explain the increasing in the pK_a value of N-1 and N-3" of netilmicin and N-3" of sisomicin (see Fig. 4.1 and Table 4.47) (Krężel et al., 2004).

Table 4.46 Comparison of the average pK_a values for aminoglycosides with the number of amino groups of particular types indicated

Aminoglycoside	**1°**	**2°**	**$R\text{-}CH_2NH_2$**	**Side-chain amino group**	**Guanidine groups**
p$K_{a\ (av)}$	7.53	8.32	8.91	9.89	12.56

1°. Primary amine

2°. Secondary amine

p$K_{a\ (av)}$. The average pK_a values determined using ^{1}H, ^{13}C, and ^{15}N NMR spectroscopy

Table 4.47 The average pK_a values of individual nitrogen atoms of the indicated aminoglycosides using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy

Individual nitrogen atoms pK_a / aminoglycoside	N-1	N-3	N-2'	N-6'	N-3''	N-2'''	N-4'''	N-6'''
neomycin C	8.08	6.86	7.98	8.65	-	8.03	-	8.76
paromomycin	8.11	6.50	8.06	-	-	8.10	-	9.08
tobramycin	7.55	6.70	7.75	9.10	7.68	-	-	-
kanamycin B	8.10	6.78	7.36	8.97	7.65	-	-	-
amikacin	-	7.64	-	8.81	8.05	-	9.89	-
sisomicin	7.42	6.22	8.00	9.30	8.50	-	-	-
netilmicin	8.15	6.52	8.14	9.29	8.47	-	-	-

p$K_{a\ (av)}$. The average pK_a values using ^{1}H, ^{13}C, and ^{15}N NMR spectroscopy

In the case of streptomycin (10), streptomycin has two guanidine groups that are more basic than side-chain amino groups, primary aminomethylene groups, and primary amines attached directly to the aminosugar rings. The stability of the conjugate acid of guanidine groups could explain this phenomenon.

It is clearly seen that its first protonation will begin at high pH, ~15 (1%), on guanidine N-1. Then becoming more protonated, 10% at pH ~14, and reaching 50% protonated at the pK_a value of guanidine N-1, pK_a = 13.06. This functional group is therefore found to be 10-fold more basic than guanidine N-3 with its pK_a = 12.06. The reasons behind this different basicity possibly include that the inductive effect of the ether group on C-4 might decrease the pK_a value of guanidine N-3, taken together with guanidine N-1 being less sterically hindered than guanidine N-3. The second (N-3) and third (N-2') protonations of streptomycin (10) are highly separated, each occurring to 50% at pH ~12.0 and ~8.0 respectively, as the secondary amino group (N-2') (pK_a = 8.16) is 10,000-fold less basic than a guanidine group (section 4.1.10 and Fig. 4.67) (Orgován and Noszál, 2012).

The presence or absence of substituents (e.g. OH) is not just affecting the NMR chemical shifts, but also affecting the ionisation constants of amino groups located on the aminoglycosides. For example, the presence of a hydroxyl group on C-3' of kanamycin B (6) causes a decrease in the pK_a of N-2', pK_a = 7.75 of tobramycin (5) to 7.36 of N-2' of kanamycin B (Fig. 4.68).

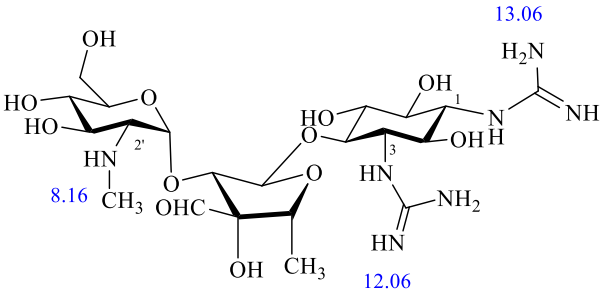
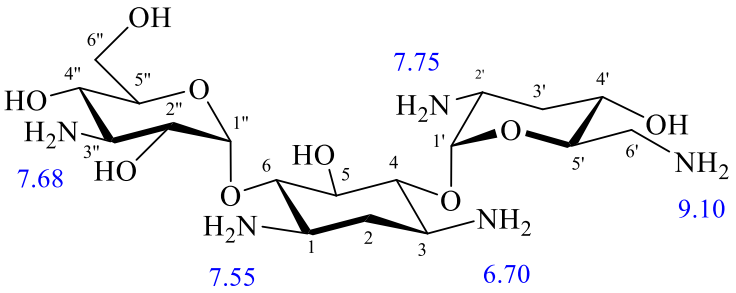


Fig. 4.67 The average pK_a values, using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopic data, of the two guanidine groups (N-1 and N-3) and the secondary amine N-2' of streptomycin

A



B

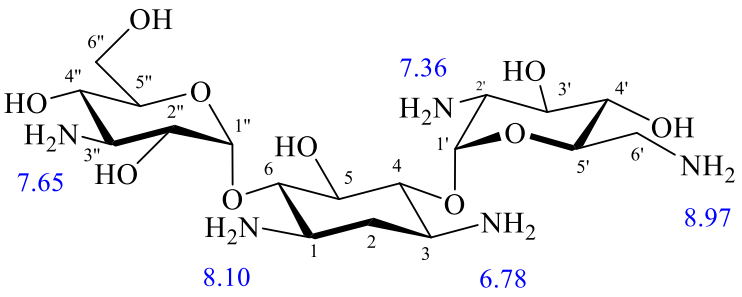


Fig. 4.68 The average pK_a values, using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopic data, of individual nitrogen atoms of A) tobramycin and B) kanamycin B

4.4. Selective reactions of tobramycin with Boc and Cbz protecting groups, and FITC

One of the ultimate objectives is to be able to trace an aminoglycoside, e.g., tobramycin (5), in the body in a non-invasive way by tagging the aminoglycoside with a fluorescent probe. The use of fluorescent derivatives of aminoglycosides may provide an insight to understand their mechanisms of toxicity. However, random FITC-labelling may present some obstacles in that it may prevent the clinical effectiveness of the aminoglycoside by labelling a crucial amine functional group and thereby blocking their biological activity. Therefore, this study aims to investigate a few specific or selective reactions of different amines located around tobramycin with amino acid protecting groups and with FITC.

Taking both the pK_a values (basicity) of amino groups and steric hindrance factors into consideration, the reaction of tobramycin (5) with 5 equiv. of $(Boc)_2O$ was carried out following the protocols in Michael et al. (1999) replacing neomycin with tobramycin. The desired penta-Boc compound 12 was obtained, as evidenced by HRMS: found 990.5203, $C_{43}H_{77}NaN_5O_{19}$ requires 990.5102 $[M + Na]^+$. The reaction of tobramycin (5) with 4 equiv. of $(Boc)_2O$ was carried out following Michael et al. (1999) where tobramycin (5) was used instead of neomycin at a temperature of 0℃ instead of 60℃ (Fig. 4.69). The yield of tetra-Boc compound 13 was 31%, because the product of the reaction of tobramycin (5) with 4 equiv. of $(Boc)_2O$ was a mixture of compounds, penta-Boc 12 (32%) and tetra-Boc 13 (31%) displaying HRMS: found 868.491, $C_{38}H_{70}N_5O_{17}$ requires 868.471 $[M + H]^+$.

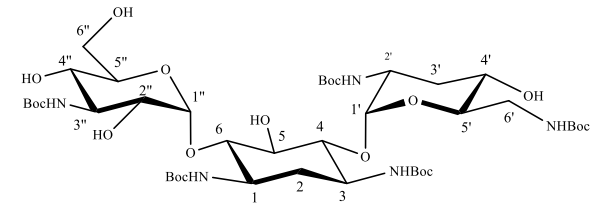
The reaction of 1 equiv. of $(Boc)_2O$, using the same conditions for compound 13, was carried out on tobramycin (5), but this did not yield any of the desired compound 14. However, using 1 equiv. of *O*-Cbz-*N*-hydroxyphthalimide, following the protocols of Chandrika et al. (2015) at a temperature of 0℃ rather than at 20℃, was created with tobramycin (5) (see Fig. 4.70). The desired compound 15 was detected using HRMS, however, due to the high polarity of compound 15, the separation process was difficult.

The comparison between ^{1}H and ^{1}H-^{13}C HSQC NMR spectra of tobramycin (5), ', and compound 13 confirm that four out of five amines were protected with Boc groups on compound 13 as expected (see Figs. 4.71, 4.72, 4.73, 4.74, 4.75, and 4.76).

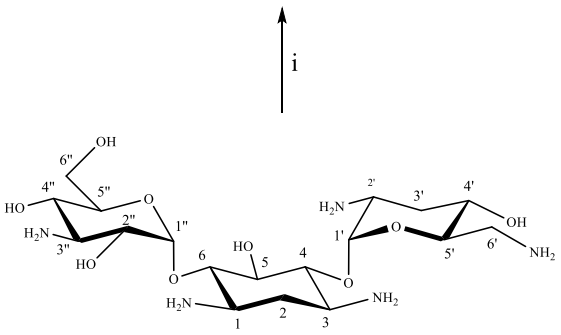
Tables 4.48, 4.49 and Fig. 4.77 show ^{1}H and ^{13}C chemical shifts and chemical shift differences ($\Delta\ \delta$) between tobramycin (5) and penta-Boc compound 12, and tobramycin (5) and tetra-Boc compound 13. The ^{1}H and ^{13}C chemical shift differences ($\Delta\ \delta$) of H-1, H-2', H-6', H-3" and C-1, C-2', C-6', C-3" of tobramycin (5) and compound 12, and tobramycin (5) and compound 13 are similar. However, the ^{1}H and ^{13}C chemical shift differences ($\Delta\ \delta$) of H-3 and C-3 of tobramycin (5) and compound 13 were obviously less than the ^{1}H and ^{13}C chemical shift differences ($\Delta\ \delta$) of H-3 and C-3 of tobramycin (5) and compound 12. This strongly suggests that the unprotected amine of compound 13 was N-3.

The explanation for this might be that the average pK_a value of N-3 using ^{1}H, ^{13}C and ^{15}N NMR spectroscopy is 6.70 which is the lowest pK_a value among those amines on tobramycin (5) (N-1, N-2', N-6' and N-3"), in other words, the basicity of N-3 is 251-times less than N-6' and 10-times less than N-1, N-2' and N-3". Moreover, the N-3 is sterically more hindered than other amines on tobramycin (5).

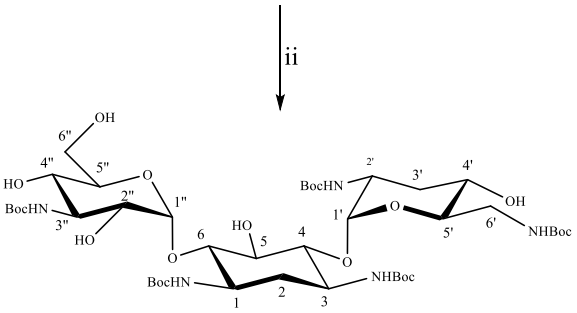
The reactions of compound 13 with FITC were attempted, following the protocols in Bandyopadhyay et al. (2017) for condition (i), except that the temperature of 80 °C for 27h was followed instead with a room temperature for 1h, and Litovchick al. (2001) for condition (ii), but this did not yield any of the desired fluorescent conjugate compound 16 (see Fig. 4.78). The possible explanation of this outcome is that the NH_2-3 is too sterically hindered. Consequently, there was no access for FITC to react with NH_2-3.



12 (80%)

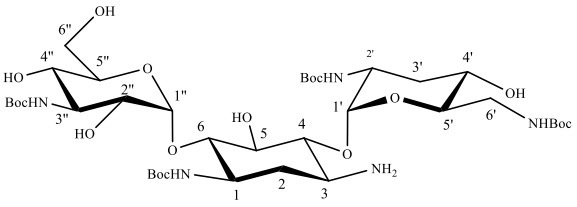


tobramycin (5)



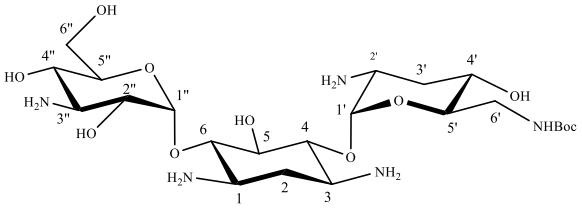
12 (32%)

+



13 (31%)

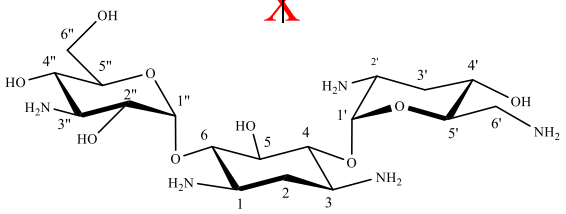
Fig. 4.69 Reagents and conditions (i) DMF, H_2O, Et_3N, $(Boc)_2O$ (5 equiv.), 60°C for 24h, 80% and (ii) DMF, H_2O, Et_3N, $(Boc)_2O$ (4 equiv.), 0°C for 2h and 20°C for 22h, 12 (32%) and 13 (31%)



14

i

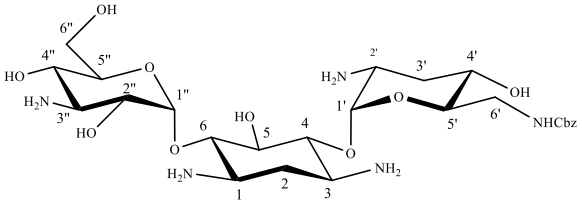
X



tobramycin (5)

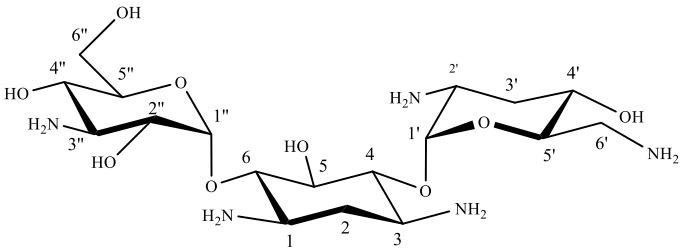
X

ii



15

Fig. 4.70 Reagents and conditions (i) DMF, H_2O, Et_3N, $(Boc)_2O$ (1 equiv.) 0°C for 2h and 20°C for 22h, 0% and (ii) MeOH: H_2O (1:1), K_2CO_3, *O*-Cbz-*N*-hydroxyphthalimide (1 equiv.) 0°C for 1h and 20 °C for 22h, 0%



tobramycin (5)

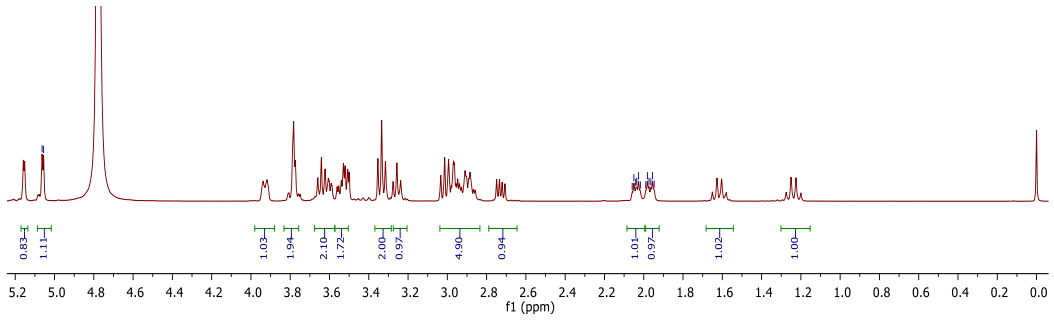


Fig. 4.71 The ^{1}H NMR spectrum of tobramycin (5)

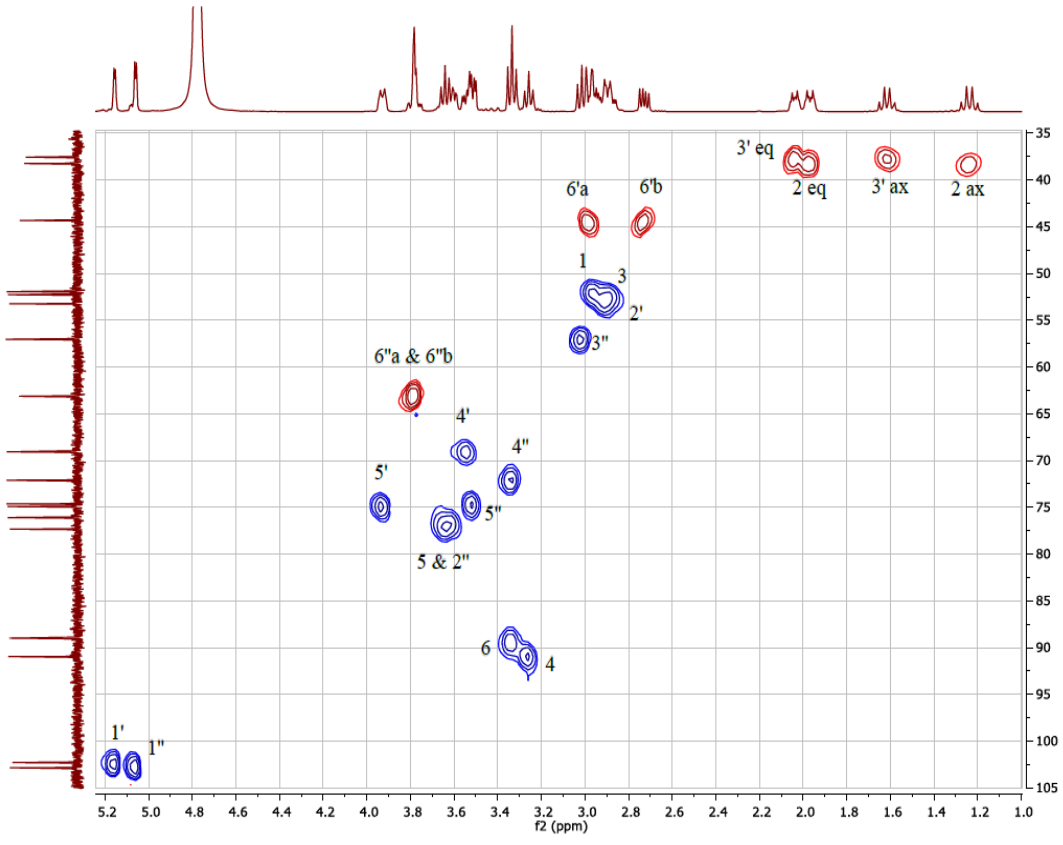
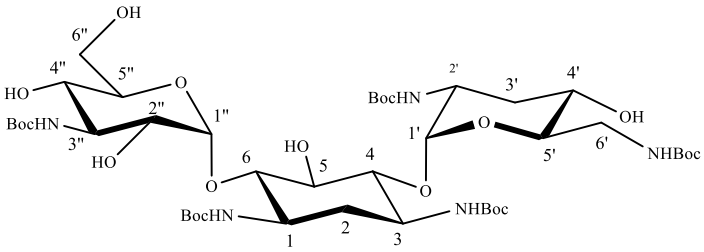


Fig. 4.72 The ^{1}H-^{13}C HSQC NMR spectrum of tobramycin (5)



compound 12

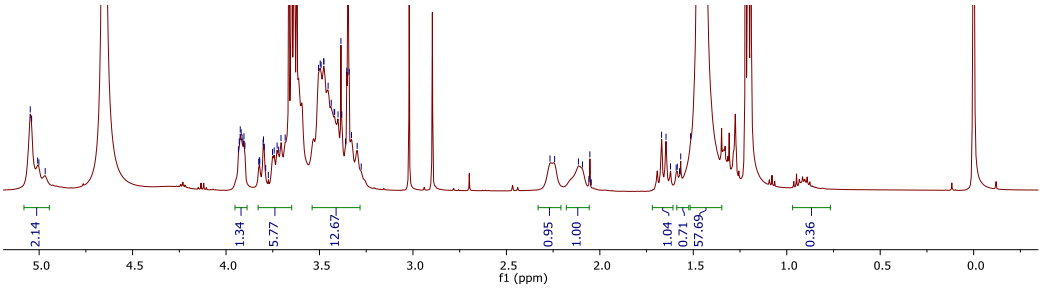


Fig. 4.73 The ^{1}H NMR spectrum of compound 12

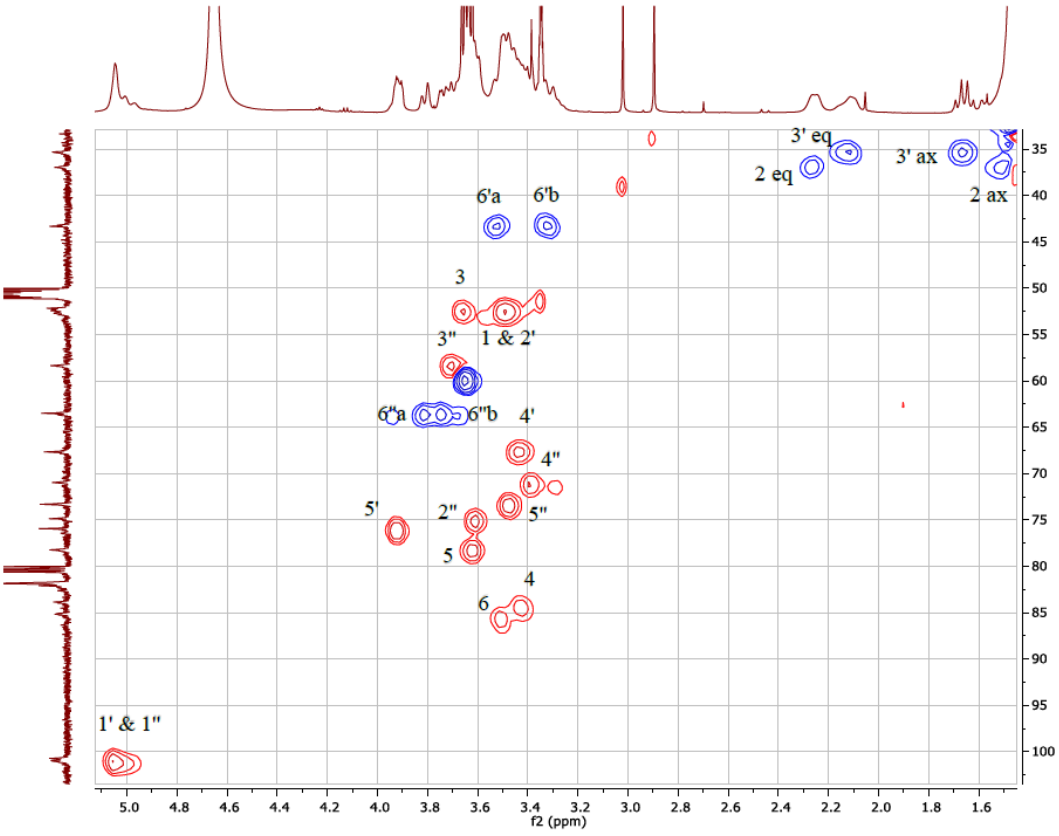
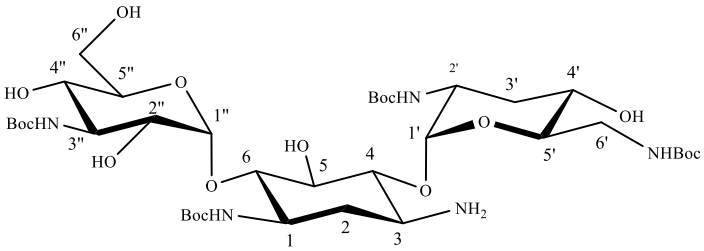


Fig. 4.74 The ^{1}H-^{13}C HSQC NMR spectrum of compound 12



compound 13

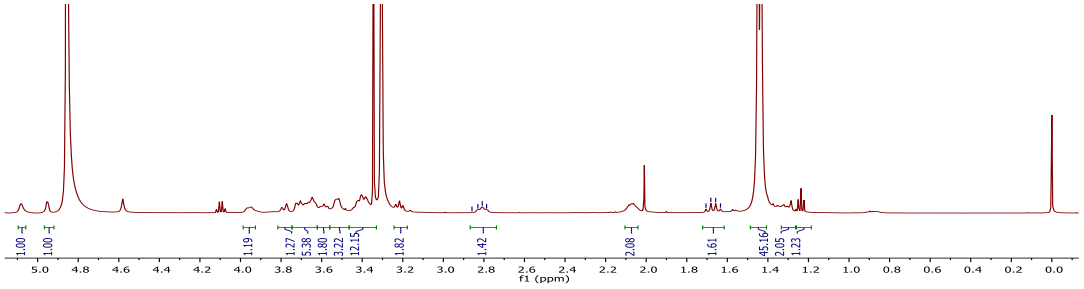


Fig. 4.75 The ^{1}H NMR spectrum of compound 13

Fig. 4.76 The ^{1}H-^{13}C HSQC NMR spectrum of compound 13

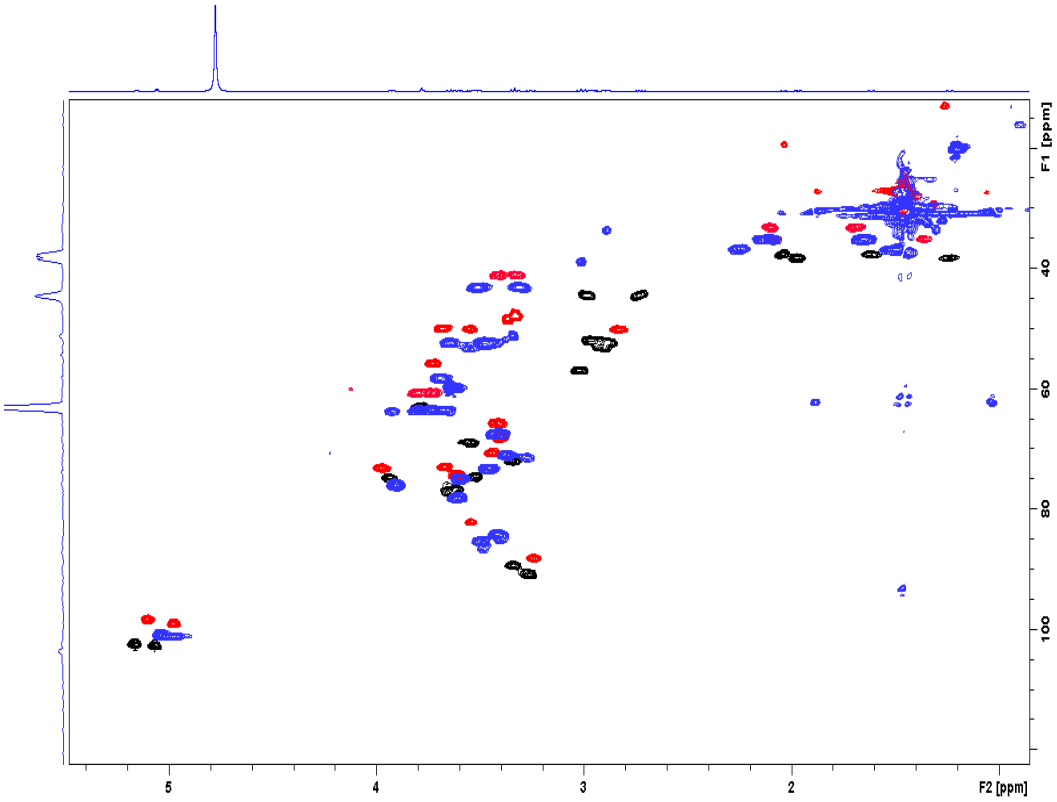


Fig. 4.77 Overlaid ^{1}H-^{13}C HSQC spectra for tobramycin (5 – in black), 12 (blue) and 13 (red) showing the changes in chemical shifts as a result of Boc protection

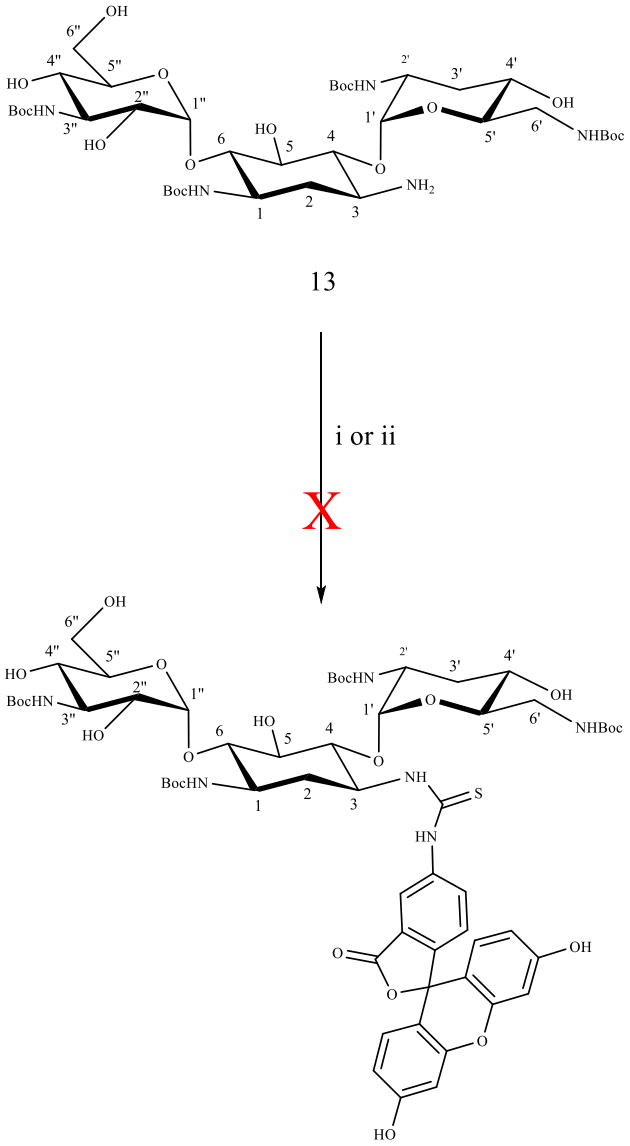


Fig. 4.78 Reagents and conditions (i) water/methanol/dioxane (1:1:1, v/v/v), FITC (2 equiv.) 60°C for 24h, 0% or (ii) DMF, Et_3N, FITC (2 equiv.) 80°C for 72h, 0%

Table 4.48 ^{1}H and ^{13}C chemical shift differences ($\Delta\ \delta$) between tobramycin (5) and compound 12. The red colour highlights the protons and carbons with nitrogen substituents.

	^{1}H and ^{13}C $\Delta\ \delta$ between tobramycin (5) and compound 12	
	^{1}H $\Delta\ \delta$	**^{13}C $\Delta\ \delta$**
1	0.58	0.71
2eq	0.30	1.48
2ax	0.26	-
3	0.67	0.46
4	0.13	4.22
5	0.04	0.43
6	0.23	4.74
1'	0.17	0.86
2'	0.57	0.24
3'eq	0.06	2.59
3'ax	0.03	-
4'	0.13	1.44
5'	0.01	1.12
6'a	0.48	1.12
6'b	0.52	-
1"	0.06	1.08
2"	0.04	1.06
3"	0.68	1.18
4"	0.04	1.29
5"	0.05	1.07
6"a	0.01	0.46
6"b	0.05	-

Table 4.49 ^{1}H and ^{13}C chemical shift differences ($\Delta\ \delta$) between tobramycin (5) and compound 13. The red colour highlights the protons and carbons with nitrogen substituents.

	^{1}H and ^{13}C $\Delta\ \delta$ between tobramycin (5) and compound 13	
	$^{1}H\ \Delta\ \delta$	**$^{13}C\ \Delta\ \delta$**
1	0.63	0.79
2eq	0.09	1.50
2ax	0.06	-
3	0.16	0.06
4	0.08	1.02
5	0.06	1.70
6	0.25	5.76
1'	0.12	2.67
2'	0.65	0.21
3'eq	0.01	2.81
3'ax	0.03	-
4'	0.15	2.01
5'	0.02	0.57
6'a	0.35	1.30
6'b	0.51	-
1"	0.14	1.42
2"	0.01	1.54
3"	0.68	1.20
4"	0.04	2.90
5"	0.10	2.67
6"a	0.01	1.21
6"b	0.08	-

Conclusions

^{1}H, ^{13}C, and ^{15}N NMR spectroscopy is a powerful technique for the measurement of individual pK_a values. Unambiguous assignments have been made for each individual amine substituent on 2-deoxystreptamine (1), neamine (2), neomycin C (3), paromomycin (4), tobramycin (5), kanamycin B (6), netilmicin (7), sisomicin (8), amikacin (9), and streptomycin (10) using variations in the chemical shift (δ) with ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy. The average pK_a values using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopic data for amines on 2-deoxystreptamine (1), neamine (2), neomycin C (3), paromomycin (4), tobramycin (5), kanamycin B (6), for netilmicin (7), sisomicin (8), amikacin (9), and streptomycin (10) are shown in Fig. 4.79. Due to its sensitivity, ^{1}H NMR spectroscopy is less time consuming (2 min for each sample) than ^{13}C (30 min for each sample) and ^{15}N HMBC (45 min for each sample) NMR spectroscopy. Consequently, ^{1}H NMR spectroscopy is the most preferable method for measuring individual pK_a values.

Taking both the pK_a values (basicity) of amino groups of tobramycin and steric hindrance factors into consideration, the investigation of the specific or selective reactions of the different amines located around tobramycin with amino acid protecting groups, e.g., Boc and Cbz, and with a fluorophore (FITC) that would generate a biologically relevant tagged tobramycin were carried out. The ^{1}H and ^{13}C NMR spectroscopic data of compounds 12 and 13 confirm that four out of five amines on tobramycin (5) were protected with Boc groups and the unprotected (unreacted) amine was N-3, which has the lowest pK_a value. However, the mono-protections of tobramycin (5) with Boc or Cbz groups and labelling the unprotected amine (N-3) on tetra-Boc compound 13 with FITC were not successful.

These results demonstrate amine basicity, and therefore reactivivity, can be predicted by NMR techniques, which can lead to selective functionalisation. One of the ultimate objectives is to be able to trace an aminoglycoside in the body in a non-invasive way by tagging the aminoglycoside with a fluorescent probe. Using the techniques described in this report, we can predict and synthesise specific FITC-labeled fluorescent derivatives of aminoglycosides that may provide an insight to understand their mechanisms of toxicity.

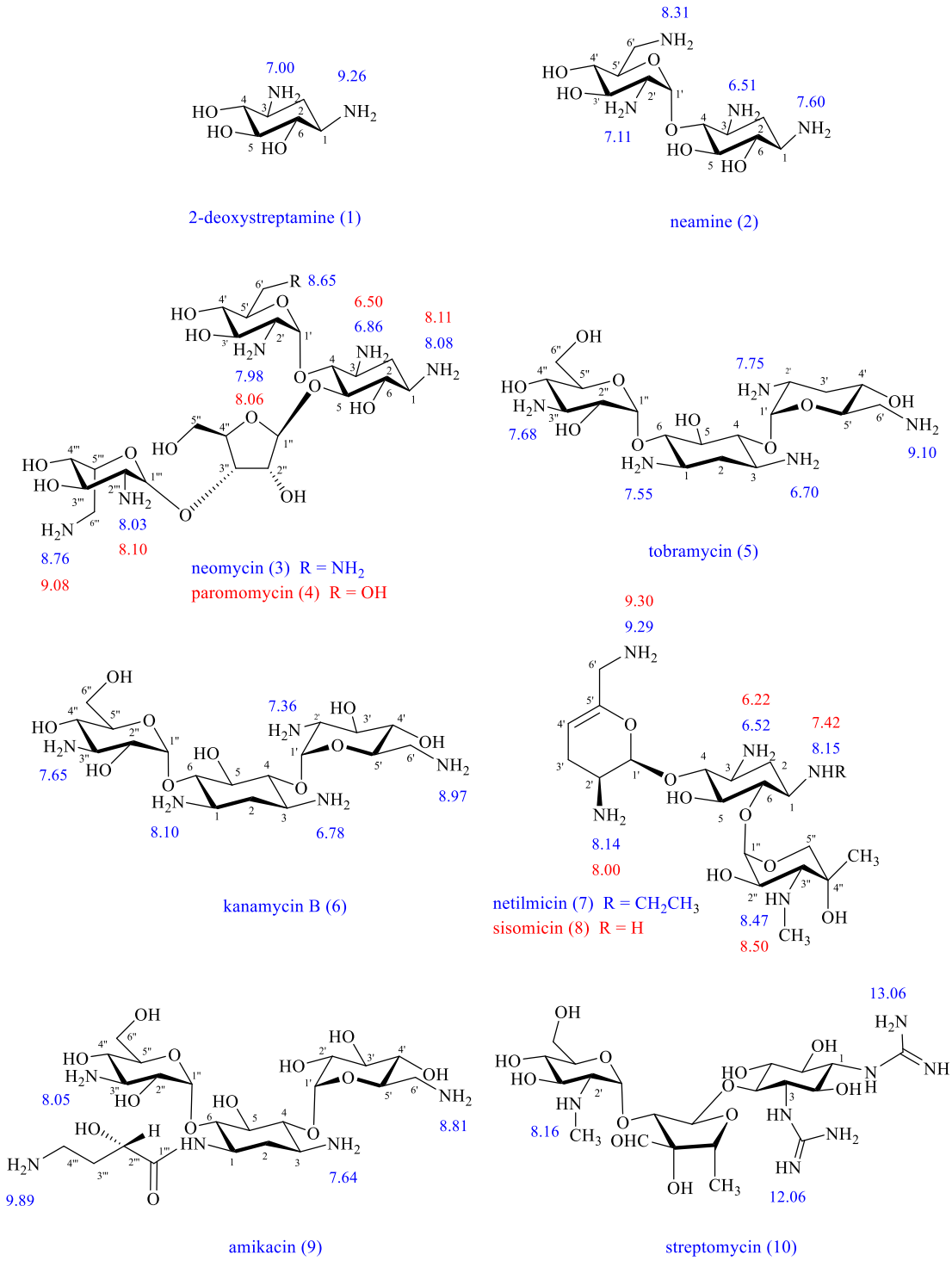


Fig. 4.79 The average pK_a values of individual nitrogen atoms of 2-deoxystreptamine (1), neamine (2), neomycin C (3), paromomycin (4), tobramycin (5), kanamycin B (6), netilmicin (7), sisomicin (8), amikacin (9), and streptomycin (10) determined using ^{1}H, ^{13}C, and ^{15}N HMBC NMR spectroscopy

References (in the style of Harvard-Bath)

- Ahmad, S. and Mokaddas, E, 2014. Current status and future trends in the diagnosis and treatment of drug-susceptible and multidrug-resistant tuberculosis. *J. Infect. Public Health*, 7(2), 75-91.
- Albert, A. and Serjeant, E.P., 1984. *The Determination of Ionisation Constants: A Laboratory Manual*. 3rd ed. London, Chapman and Hall, 155-172.
- Alekseev, V.G. and Markova, E.V., 2016. Constants of acid–base equilibria in an aqueous amikacin aminoglycoside solution at 298 K. *Russ. J. Phys. Chem. A*, 90(3), 586-591.
- Andac, C.A., Stringfellow, T.C., Hornemann, U. and Noyanalpan, N., 2011. NMR and amber analysis of the neamine pharmacophore for the design of novel aminoglycoside antibiotics. *Bioorg. Chem.*, 39(1), 28-41.
- Armstrong, E.S., Kostrub, C.F., Cass, R.T., Moser, H.E., Serio, A.W. and Miller, G.H., 2012. Aminoglycosides. In: T.J. Dougherty, and M.J. Pucci, ed. *Antibiotic Discovery and Development*. 2nd ed. Boston, Springer, 229-270.
- Asghar, A.H. and Ahmad, O.B., 2018. Prevalence of aminoglycoside resistance genes in *Pseudomonas aeruginosa* isolated from a tertiary care hospital in Makkah, KSA. *Pak. J. Med. Sci.*, 15(2), 541-547.
- Avdeef, A., 2012. *Absorption and Drug Development: Solubility, Permeability, and Charge State*. 2nd ed. New Jersey, John Wiley & Sons, 35-40.
- Avent, M., Rogers, B., Cheng, A. and Paterson, D., 2011. Current use of aminoglycosides: indications, pharmacokinetics and monitoring for toxicity. *Intern. Med. J.*, 41(6), 441-449.
- Balakumar, P., Rohilla, A. and Thangathirupathi, A., 2010. Gentamicin-induced nephrotoxicity: Do we have a promising therapeutic approach to blunt it? *Pharmacol. Res.*, 62(3), 179-186.

- Bandyopadhyay, A., Cambray, S. and Gao, J., 2017. Fast diazaborine formation of semicarbazide enables facile labelling of bacterial pathogens. *J. Am. Chem. Soc.*, 139(2), 871-878.
- Baran, Y., Kau, P.M., Lawrance, G.A. and Von Nagy-Felsobuki, E.I., 2001. Interactions of the aminoglycoside neamine and 2-deoxystreptamine with copper (II) and zinc (II). *Bioinorg. React. Mech.*, 3(1), 31-38.
- Barbieri, C.M. and Pilch, D.S., 2006. Complete thermodynamic characterization of the multiple protonation equilibria of the aminoglycoside antibiotic paromomycin: a calorimetric and natural abundance ^{15}N NMR study. *Biophys. J.*, 90(4), 1338-1349.
- Beale, J.M., Block, J. and Hill, R., 2010. *Organic Medicinal and Pharmaceutical Chemistry.* 12th ed. Philadelphia. Lippincott, Williams & Wilkins, 294-300.
- Becker, B. and Cooper, M., 2013. Aminoglycoside antibiotics in the 21st century. *ACS Chem. Bio.*, 8(1), 105-115.
- Benveniste, R. and Davies, J., 2016. Structure-activity relationships among the aminoglycoside antibiotics: role of hydroxyl and amino groups. *Antimicrob. Agents Chemoth.*, 4(4), 402-409.
- Bera, S., Zhanel, G. and Schweizer, F., 2010. Antibacterial activity of guanidinylated neomycin B- and kanamycin A-derived amphiphilic lipid conjugates. *J. Antimicrob. Chemoth.*, 65(6), 1224-1227.
- Bezençon, J., Wittwer, B., Cutting, B., Smieško, M., Wagner, B., Kansy, M. and Ernst, B., 2014. pK_a determination by ^{1}H NMR spectroscopy – An old methodology revisited. *J. Pharmaceut. Biomed.*, 93(6), 147-155.
- Blagbrough, I.S., Metwally, A.A. and Geall, A.J., 2011. Measurement of polyamine pK_a values. In: A.E. Pegg, and R.A. Casero, ed. *Polyamines*. New York, Humana Press, 493-503.

- Botto, R.E. and Coxon, B., 1983. Nitrogen-15 nuclear magnetic resonance spectroscopy of neomycin B and related aminoglycosides. *J. Am. Chem. Soc.*, 105(4), 1021-1028.
- Box, K.J., Donkor, R.E., Jupp, P.A., Leader, I.P., Trew, D.F. and Turner, C.H., 2008. The chemistry of multi-protic drugs: Part 1: A potentiometric, multi-wavelength UV, and NMR pH titrimetric study of the micro-speciation of SKI-606. *Pharmaceut. Biomed.*, 47(2), 303-311.
- Brenner, G. and Stevens, C., 2013. *Pharmacology*. 4th ed. Philadelphia, Saunders/Elsevier, 425-451.
- Burton, M.E., 2006. *Applied Pharmacokinetics and Pharmacodynamics: Principles of Therapeutic Drug Monitoring*. 4th ed. Philadelphia, Lippincott Williams and Wilkins, 285-325.
- Carpenter, B., Feese, E., Sadeghifar, H., Argyropoulos, D. and Ghiladi, R., 2012. Porphyrin-cellulose nanocrystals: A photobactericidal material that exhibits broad spectrum antimicrobial activity. *Photochem. Photobiol.*, 88(3), 527-536.
- Cojocel, C. and Hook, J.B., 1983. Aminoglycoside nephrotoxicity. *Trends in Pharmacol. Sci.*, 4(3), 174-179.
- Cook, W.G. and Lister, D.H., 2014. Chemistry in CANDU process systems. In: B. Garland, ed. *The Essential CANDU*. Hamilton, Ontario: UNENE, 5-7.
- Cox, J.R. and Serpersu, E.H., 1997. Biologically important conformations of aminoglycoside antibiotics bound to an aminoglycoside 3'-phosphotransferase as determined by transferred nuclear overhauser effect spectroscopy. *Biochem.*, 36(9), 2353-2359.
- Cragg, G.M. and Newman, D.J., 2001. Natural product drug discovery in the next millennium. *Pharma. Biol.*, 39(1), 8–17.
- De Levie, R., 2003. The Henderson-Hasselbalch equation: its history and limitations. *Chem. Educ.*, 80(2), 146-151.

- Dorman, D.E., Paschal, J.W. and Merkel, K.E., 1976. Nitrogen-15 nuclear magnetic resonance spectroscopy. The nebramycin aminoglycosides. *J. Am. Chem. Soc.*, 98(22), 6885-6888.
- Fărcaşiu, D. and Ghenciu, A., 1996. Determination of acidity functions and acid strengths by ^{13}C NMR. *Prog. Nucl. Mag. Res. Sp.*, 29(3), 129-168.
- Farnsworth, N.R., Akerele, O., Bingel, A.S., Soejarto, D.D. and Guo, Z., 1985. Medicinal plants in therapy. *B. World Health Organ.*, 63(6), 965-969.
- FDA, 2018. https://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/Anti-InfectiveDrugsAdvisoryCommittee/UCM611379.pdf
- Foye, W., Lemke, T. and Williams, D., 2008. *Foye's Principles of Medicinal Chemistry.* 6th ed. Philadelphia*:* Lippincott Williams and Wilkins, 1028-1083.
- François, B., Russell, R.J., Murray, J.B., Aboul-ela, F., Masquida, B., Vicens, Q. and Westhof, E., 2005. Crystal structures of complexes between aminoglycosides and decoding A site oligonucleotides: role of the number of rings and positive charges in the specific binding leading to miscoding. *Nucleic Acids Res.*, 33(17), 5677-5690.
- Frassineti, C., Ghelli, S., Gans, P., Sabatini, A., Moruzzi, M.S. and Vacca, A., 1995. Nuclear magnetic resonance as a tool for determining protonation constants of natural polyprotic bases in solution. *Anal. Biochem.*, 231(2), 374-382.
- Freire, F., Cuesta, I., Corzana, F., Revuelta, J., González, C., Hricovini, M., Bastida, A., Jiménez-Barbero, J. and Asensio, J.L., 2007. A simple NMR analysis of the protonation equilibrium that accompanies aminoglycoside recognition: dramatic alterations in the neomycin-B protonation state upon binding to a 23-mer RNA aptamer. *Chem. Commun.*, 34(2), 174-176.
- Fuentes-Martinez, J.P., Gutiérrez-Rodrigueza, D., Garcia, E.R., Rivera-Mirqueza, K.I., Medrano, F., Torres-Angeles, O., Castillo-Vargas, E., Duque, B.M. and Godoy-

Alcántar, C., 2014. Streptomycin hydrazone derivatives: synthesis and molecular recognition in aqueous solution. *Nat. Prod. Commun.*, 9(10), 1449-1455.

- Fuentes-Martínez, Y., Godoy-Alcántar, C., Medrano, F., Dikiy, A. and Yatsimirsky, A.K., 2010a. Protonation of kanamycin A: Detailing of thermodynamics and protonation sites assignment. *Bioorg. Chem.*, 38(4), 173-180.
- Fuentes-Martínez, Y., Godoy-Alcántar, C., Medrano, F., Dikiy, A. and Yatsimirsky, A.K., 2010b. Nucleotide recognition by protonated aminoglycosides. *Supramol. Chem.*, 22(4), 212-220.
- Gad, S.C., 2005. *Drug Discovery Handbook. New Jersey*, John Wiley & Sons Inc, 11-70.
- Gaggelli, E., Gaggelli, N., Maccotta, A., Valensin, G., Marini, D., Di Cocco, M.E. and Delfini, M., 1995. Determination of intramolecular hydrogen bonds in amikacin in water solution by NMR spectroscopy. *Spectrochim. Acta, A*, 51(11), 1959-1963.
- Gift, A., Stewart, S. and Bokashanga, P., 2012. Experimental determination of pK_a values by use of NMR chemical shifts, Revisited. *J. Chem. Educ.*, 89(11), 1458-1460.
- Grunwald, E., Loewenstein, A. and Meiboom, S., 1957. Application of nuclear magnetic resonance to the study of acid-base equilibria. *J. Chem. Phys.*, 27(3), 641-642.
- Guldberg C.M. and Waage P., 1864. Studies concerning affinity. *Videnskabs-Selsk.*, 92(2), 35-41.
- Gutiérrez-Moreno, N.J., Medrano, F. and Yatsimirsky, A.K., 2012. Schiff base formation and recognition of amino sugars, aminoglycosides and biological polyamines by 2-formyl phenylboronic acid in aqueous solution. *Org. Biomol. Chem.*, 10(34), 6960-6972.
- Harvey, S.C. and Skolnick, P., 1999. Polyamine-like actions of aminoglycosides at recombinant *N*-methyl-D-aspartate receptors. *J. Pharmacol. Exp. Ther.*, 291(1), 285-291.

- Hasselbalch, K.A., 1916. Die berechnung der wasserstoffzahl des blutes aus der freien und gebundenen kohlensäure desselben, und die sauerstoffbindung des blutes als funktion der wasserstoffzahl. *Biochem.*, 78(1), 112-114.
- Hegde, R., Borkow, G., Berchanski, A. and Lapidot, A., 2007. Structure–function relationship of novel X4 HIV-1 entry inhibitors – L-and D-arginine peptide-aminoglycoside conjugates. *FEBS J.*, 274(24), 6523-6536.
- Heidary, N. and Cohen, D., 2005. Hypersensitivity reactions to vaccine components. *Dermatitis*, 16(03), 115-117.
- Henderson, L.J., 1908. Concerning the relationship between the strength of acids and their capacity to preserve neutrality. *Am. J. Physiol.*, 21(2), 173-179.
- Herzog, I., Green, K., Berkov-Zrihen, Y., Feldman, M., Vidavski, R., Eldar-Boock, A., Satchi-Fainaro, R., Eldar, A., Garneau-Tsodikova, S. and Fridman, M., 2012. 6″-Thioether tobramycin analogues: towards selective targeting of bacterial membranes. *Angew. Chem.*, 124(23), 5750-5754.
- Hobbie, S.N., Pfister, P., Bruell, C., Sander, P., François, B., Westhof, E. and Böttger, E.C., 2006. Binding of neomycin-class aminoglycoside antibiotics to mutant ribosomes with alterations in the A site of 16S rRNA. *J. Antimicrob. Agents Chemoth.*, 50(4), 1489-1496.
- Hofer, U., 2013. Antimicrobials: Aminoglycosides flip the switch on resistance. *Nat. Rev. Microbiol.*, 11(3), 149-149.
- Holmes, W.C. and Snyder, E.F., 1925. The spectrophotometric determination of hydrogen-ion concentrations and of the apparent dissociation constants of indicators. *J. Am. Chem. Soc.*, 47(1), 226-229.
- Hong, S.H., Park, S.K., Cho, Y.S., Lee, H.S., Kim, K.R., Kim, M.G. and Chung, W.H., 2006. Gentamicin induced nitric oxide-related oxidative damages on vestibular afferents in the guinea pig. *Hearing Res.*, 211(1), 46-53.

- Hooper, I., 1982. The naturally occurring aminoglycosides antibiotics. In: H. Umezawa, and I. Hooper, ed. *Aminoglycoside Antibiotics*. Berlin, Heidelberg, Springer Berlin Heidelberg, 1-10.
- Hörter, D. and Dressman, J.B., 2001. Influence of physicochemical properties on dissolution of drugs in the gastrointestinal tract. *Adv. Drug Deliver. Rev.*, 46(1), 75-87.
- Houghton, J., Green, K., Chen, W. and Garneau-Tsodikova, S., 2010. The future of aminoglycosides: the end or renaissance? *Bio. Chem.*, 11(7), 880-902.
- Inouye, S., 1968. On the prediction of pK_a values of amino sugars. *Chem. Pharm. Bull.*, 16(6), 1134-1137.
- Janknegt, R., 1990. Aminoglycoside therapy. *Pharm. World Sci.*, 12(3), 81-90.
- Jeżowska-Bojczuk, M. and Bal, W., 1998. Co-ordination of copper (II) by amikacin. Complexation equilibria in solution and oxygen activation by the resulting complexes. *J. Chem. Soc. Dalton Trans.*, 13(1), 153-160.
- Jeżowska-Bojczuk, M., Karaczyn, A. and Kozłowski, H., 1998. Copper (II) binding to tobramycin: potentiometric and spectroscopic studies. *Carbohyd. Res.*, 313(3-4), 265-269.
- Jeżowska-Bojczuk, M., Szczepanik, W., Mangani, S., Gaggelli, E., Gaggelli, N. and Valensin, G., 2005. Identification of copper (II) binding sites in the aminoglycosidic antibiotic neomycin B. *Inorg. Chem.*, 6(15), 3063-3071.
- Jiang, L. and Patel, D.J., 1998. Solution structure of the tobramycin–RNA aptamer complex. *Nat. Struct. Mol. Biol.*, 5(9), 769.
- Joint Formulary Committee, 2018. *British National Formulary*. Available at: http://www.medicinescomplete.com (Accessed: 20/11/2018).
- Kaul, M., Barbieri, C., Kerrigan, J. and Pilch, D., 2003. Coupling of drug protonation to the specific binding of aminoglycosides to the site of 16s rRNA: elucidation of the

number of drug amino groups involved and their identities. *Mol. Biol.*, 326(5), 1373-1387.

- Kling, D., Hesek, D., Shi, Q. and Mobashery, S., 2007. Design and synthesis of a structurally constrained aminoglycoside. *Org. Chem.*, 72(14), 5450-5453.
- Kong, B., Joshi, T., Belousoff, M.J., Tor, Y., Graham, B. and Spiccia, L., 2016. Neomycin B-cyclen conjugates and their Zn (II) complexes as RNA-binding agents. *J. Inorg. Biochem.*, 162(5), 334-342.
- Krężel, A., Szczepanik, W., Świątek, M. and Jeżowska-Bojczuk, M., 2004. Acid–base versus structural properties of an aminoglycoside antibiotic – sisomicin: NMR and potentiometric approach. *Bioorg. Med. Chem.*, 12(15), 4075-4080.
- Kuehl, F.A., Peck, R.L., Hoffhine, C.E., Graber, R.P. and Folkers, K., 1946. Streptomyces antibiotics. VIII. Isolation of streptomycin. *J. Am. Chem. Soc.*, 68(8), 1460-1462.
- Kulik, M., Goral, A., Jasiński, M., Dominiak, P. and Trylska, J., 2015. Electrostatic interactions in aminoglycoside–RNA complexes. *Biophys. J.*, 108(3), 655–665.
- Lapidot, A., Berchanski, A. and Borkow, G., 2008. Insight into the mechanisms of aminoglycoside derivatives interaction with HIV-1 entry steps and viral gene transcription. *The FEBS J.*, 275(21), 5236-5257.
- Lebeaux, D., Leflon-Guibout, V., Ghigo, J. and Beloin, C., 2015. In vitro activity of gentamicin, vancomycin or amikacin combined with EDTA or L-arginine as lock therapy against a wide spectrum of biofilm-forming clinical strains isolated from catheter-related infections. *J. Antimicrob. Chemoth.*, 70(6), 1704-1712.
- Lesniak, W., Mc Laren, J., Harris, W.R., Pecoraro, V.L. and Schacht, J., 2003. An isocratic separation of underivatized gentamicin components, ^{1}H NMR assignment and protonation pattern. *Carbohyd. Res.*, 338(24), 2853-2862.

- Litovchick, A., Lapidot, A., Eisenstein, M., Kalinkovich, A. and Borkow, G., 2001. Neomycin B − arginine conjugate, a novel HIV-1 tat antagonist: synthesis and anti-HIV activities. *Biochemistry*, 40(51), 15612-15623.
- LoBue, P.A., 2005. Inhaled tobramycin: not just for cystic fibrosis anymore? *Chest J.*, 127(4), 1098-1101.
- Manallack, D.T., 2007. The pK_a distribution of drugs: application to drug discovery. *Perspect. Medicin. Chem.*, 1(3), 25-31.
- Maviglia, R., Nestorini, R. and Pennisi, M.A., 2009. Role of old antibiotics in multidrug resistant bacterial infections. *Curr. Drug Targets*, 10(9), 895-905.
- Michael, K., Wang, H. and Tor, Y., 1999. Enhanced RNA binding of dimerized aminoglycosides. *Bioorgan. Med. Chem.*, 7(7), 1361-1371.
- Mingeot-Leclercq, M.P. and Tulkens, P.M., 1999. Aminoglycosides: nephrotoxicity. *Antimicrob. Agents Chemoth.*, 43(5), 1003-1012.
- Mingeot-Leclercq, M.P., Glupczynski, Y. and Tulkens, P.M., 1999. Aminoglycosides: Activity and resistance. *Antimicrob. Agents Chemoth.*, 43(4), 727-737.
- Moazed, D. and Noller, H.F., 1987. Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature*, 327(6121), 389-394.
- Mousseau, G., Mediouni, S. and Valente, S.T., 2015. Targeting HIV transcription: the quest for a functional cure. In: E. Torbett, D. Goodsell, and D. Richman, ed. *The Future of HIV-1 Therapeutics*. New York, Springer, 121-160.
- Ogle, J.M. and Ramakrishnan, V., 2005. Structural insights into translational fidelity. *Annu. Rev. Biochem.*, 74(4), 129-177.
- Orgován, G. and Noszál, B., 2012. NMR analysis and site-specific protonation constants of streptomycin. *J. Pharmaceut. Biomed. Anal.*, 59(2), 78-82.
- Özen, C., Malek, J. and Serpersu, E., 2006. Dissection of aminoglycoside−enzyme interactions: A calorimetric and NMR study of neomycin B binding to the

aminoglycoside phosphotransferase (3‘)-IIIa. *J. Am. Chem. Soc.*, 128(47), 15248-15254.

- Pagano, T.G., Gong, Y., Kong, F., Tsao, R., Fawzi, M. and Zhu, T., 2011. Structural characterization of the tobramycin–piperacillin reaction product formed at pH 6.0. *Antibiotics*, 64(10), 673.
- Perez-Fernandez, D., Shcherbakov, D., Matt, T., Leong, N., Kudyba, I., Duscha, S., Boukari, H., Patak, R., Dubbaka, S., Lang, K., Meyer, M., Akbergenov, R., Freihofer, P., Vaddi, S., Thommes, P., Ramakrishnan, V., Vasella, A. and Böttger, E., 2014. 4′-O-substitutions determine selectivity of aminoglycoside antibiotics. *Nature Commun.*, 25(2), 24-33.
- Petersen, L. and Rogers, C., 2015. Aminoglycoside-induced hearing deficits – a review of cochlear ototoxicity. *S. Afr. Fam. Pract.*, 57(2), 77-82.
- Po, H.N. and Senozan, N.M., 2001. The Henderson-Hasselbalch equation: its history and limitations. *Chem. Educ.*, 78(11), 1499-1510.
- Popov, K., Rönkkömäki, H. and Lajunen, L.H., 2006. Guidelines for NMR measurements for determination of high and low pK_a values (IUPAC Technical Report). *Pure and Appl. Chem.*, 78(3), 663-675.
- Rahim, A., Sayuti, M., Hau, K., Zaki, W., Raskitar, N. and Kanasin, R., 2011. An illustrated review about aminoglycosides. *J. Pharm. Sci.*, 2(12), 2744-2746.
- Ramirez, M. and Tolmasky, M., 2010. Aminoglycoside modifying enzymes. *Drug Resist. Updates*, 13(6), 151-171.
- Reijenga, J., Van Hoof, A., Van Loon, A. and Teunissen, B., 2013. Development of methods for the determination of pK_a values. *J. Anal. Chem.*, 8(3), 304-307.
- Ricci, A., 2008. *Amino Group Chemistry: From Synthesis to the Life Sciences*. Morlenbach, John Wiley and Sons, 305-320.

- Russell, R.J., Murray, J.B., Lentzen, G., Haddad, J. and Mobashery, S., 2003. The complex of a designer antibiotic with a model aminoacyl site of the 30S ribosomal subunit revealed by X-ray crystallography. *J. Am. Chem. Soc.*, 125(12), 3410-3411.
- Schatz, A., Bugle, E. and Waksman, S.A., 1944. Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. *Exp. Biol. Med.*, 55(1), 66-69.
- Schoental, R., 1965. Toxicology of natural products. *Food Cosm. Toxicol.*, 3(2), 609–620.
- Seiler, N., Hardy, A. and Moulinoux, J.P. 1996. Aminoglycosides and polyamines: Targets and effects in the mammalian organism of two important groups of natural aliphatic polycations. *Prog. Drug Res*., 46(7), 183-241.
- Selimoglu, E., 2007. Aminoglycoside-induced ototoxicity. *Curr. Pharm. Design*, 13(1), 119-126.
- Shah, P.M., Heetderks, G. and Stille, W., 1977. Bactericidal activity of amikacin and gentamicin. *Chemotherapy*, 23(4), 260–266.
- Sutrisno, B., 2001. Determination of acid dissociation constants of neamine by potentiometric and electrospray mass spectral techniques. *Struct. Chem*, 12(3), 189-195.
- Szczepanik, W., Kaczmarek, P., Sobczak, J., Bal, W., Gatner, K. and Jeżowska-Bojczuk, M., 2002. Copper (II) binding by kanamycin A and hydrogen peroxide activation by resulting complexes. *New J. Chem.*, 26(10), 1507-1514.
- Takeda, Y., Samejima, K., Nagano, K., Watanabe, M., Sugeta, H. and Kyogoku, Y., 1983. Determination of protonation sites in thermospermine and in some other polyamines by ^{15}N and ^{13}C nuclear magnetic resonance spectroscopy. *Eur. J. Biochem.*, 130(2), 383-389.

- Thamban Chandrika, N., Green, K.D., Houghton, J.L. and Garneau-Tsodikova, S., 2015. Synthesis and biological activity of mono-and di-N-acylated aminoglycosides. *ACS Med. Chem. Lett.*, 6(11), 1134-1139.
- Tiwow, V.M., 2014. Determination of stability constants of aminoglycoside antibiotics with their metal complexes. In: *AIP Conference Proceedings*, 1589(1) 220-225.
- Vicens, Q. and Westhof, E., 2003. Molecular recognition of aminoglycoside antibiotics by ribosomal RNA and resistance enzymes: An analysis of X-ray crystal structures. *Biopolymers*, 70(1), 42-57.
- Waksman, S. and Lechevalier, H., 1949. Neomycin, a new antibiotic active against streptomycin-resistant bacteria, including tuberculosis organisms. *Science*, 109(2830), 305–307.
- Wang, H. and Tor, Y., 1997. Electrostatic interactions in RNA aminoglycosides binding. *J. Am. Chem. Soc*., 119(37), 8734-8735.
- Watkins, D., Norris, F., Kumar, S. and Arya, D., 2013. A fluorescence-based screen for ribosome binding antibiotics. *Anal. Biochem.*, 434(2), 300-307.
- Weisblum, B. and Davies, J., 1968. Antibiotic inhibitors of the bacterial ribosome. *Bacteriol. Rev.*, 32(4), 493-499.
- Whelton, A. and Neu, C., 1982. *The Aminoglycosides: Microbiology, Clinical Use, and Toxicology*. New York, Marcel Dekker Inc, 20-70.
- Wishart, D.S., Bigam, C.G., Yao, J., Abildgaard, F., Dyson, H.J., Oldfield, E., Markley, J.L. and Sykes, B.D., 1995. ^{1}H, ^{13}C and ^{15}N chemical shift referencing in biomolecular NMR. J. *Biomol. NMR*, 6(2), 135-140.
- Zeitler, K., Salvas, B., Stevens, V. and Brown, J., 2012. Aztreonam lysine for inhalation: new formulation of an old antibiotic. *Am. J. Health Syst.*, 69(2), 107-115.

- Zhang, J., Chiang, F.I., Wu, L., Czyryca, P.G., Li, D. and Chang, W.T., 2008. Surprising alteration of antibacterial activity of 5″-modified neomycin against resistant bacteria. *Med. Chem.*, 51(23), 7563-7573.
- Zhang, M. and Vogel, H.J., 1993. Determination of the side chain pK_a values of the lysine residues in calmodulin. *Biol. Chem.*, 268(30), 420-428.

Poster Abstracts

Individual pK_a values of aminoglycosides determined by different NMR spectroscopies

AbdulAziz H. Al Khzem, Timothy J. Woodman, and Ian S. Blagbrough*

Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, UK

Purpose

The amino functional group substituents around the different rings of aminoglycoside antibiotics are key to the biological activities of these natural product alkaloids. The ionisation constant (pK_a) is the pH at which functional groups are 50% ionized. The pK_a values of any medication play a significant role in the physicochemical data and are relevant to drug activity. This study is on determining individual pK_a values by detailed Nuclear Magnetic Resonance (NMR) spectroscopy of selected aminoglycoside alkaloids from *Streptomyces* and *Micromonospora*. In order to determine the individual pK_a values, not available by potentiometric methods, different NMR reporter nuclei have been employed. Studying the pK_a values of these alkaloids will afford a better understanding of their structure-activity relationships (SAR), especially the order in which these similar functional groups gain/lose protons. Such data will potentially help in understanding the order of target mRNA binding of key basic functional groups. The aim is to measure pK_a values of individual amines on aminoglycosides by using new combinations of ^{1}H, ^{13}C, and ^{15}N NMR spectroscopic data.

Methods

Aminoglycoside analyte solutions (0.73-0.15 M aminoglycoside in 99.97% D_2O) were prepared at a ~10 mg/mL concentration. NMR spectra including ^{1}H, ^{13}C, HSQC, HMBC, NOESY, and ^{15}N-HMBC were recorded on Bruker Avance III 400 and 500 MHz spectrometers. Trimethylsilylpropanoic acid (TMSP) was used as a reference for ^{1}H and ^{13}C NMR spectroscopy. ^{15}N chemical shifts were measured relative to external CH_3NO_2 set at -511.72 ppm. The pH values were adjusted using 0.5 M NaOD/DCl. MestReNova was used for analysis of the recorded spectra. The nonlinear sigmoidal curve and the inflection point of the sigmoidal curve were determined using GraphPad Prism 7 (Version 2017), after subtraction of 0.5 to convert the measured pD values into pH values.

Results

The pK_a values of 1-NH_2 and 3-NH_2 of 2-deoxystreptamine are 9.26 and 7.00. The order of ionisation constants for neamine is: N-6' (8.31) > N-1 (7.60) > N-2' (7.11) > N-3 (6.50), for neomycin is: N-6''' (8.76) > N-6' (8.65) > N-1 (8.08) ≈ N-2' (7.98) ≈ N-2''' (8.03) > N-3 (6.86), for tobramycin is: N-6' (9.10) > N-2' (7.75) ≈ N-3'' (7.68) > N-1 (7.55) > N-3 (6.70) and for sisomicin is: N-6' (9.30) > N-3'' (8.50) > N-2' (8.00) > N-1 (7.42) > N-3 (6.22).

Conclusions

^{1}H, ^{13}C, and ^{15}N NMR spectroscopy is a powerful tool for the measurement of individual pK_a values. Moreover, because of its sensitivity, ^{1}H NMR spectroscopy is less time consuming (2 min for each sample) than ^{13}C (30 min for each sample) and ^{15}N HMBC (45 min for each sample) NMR spectroscopy. Unambiguous assignments have been made for each individual amine substituent on these clinically important aminoglycoside antibiotics.

Acknowledgments

We thank the Government of the Kingdom of Saudi Arabia for fully funding this studentship.

Individual pK_a values of aminoglycosides determined by different NMR spectroscopies

AbdulAziz H. Alkhzem, Timothy J. Woodman, and Ian S. Blagbrough*
Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, UK

e-mail: prsisb@bath.ac.uk

Objectives

The objectives of these studies are to measure the individual ionisation constant (pK_a) values of each amine on selected aminoglycoside alkaloids from *Streptomyces* and *Micromonospora* by using new combinations of ^{1}H, ^{13}C, and ^{15}N-HMBC NMR spectroscopic data. The pK_a is the pH at which functional groups are 50% ionized. Any medication's pK_a values have an important role to play in their physicochemical data and they are also important in their biological activities. Various NMR reporter nuclei have been used to ascertain the individual pK_a values that cannot be obtained by potentiometric methods, particularly the order in which the amino functional groups either gain or shed protons. These data have the capacity to enhance comprehension of the order of target mRNA binding of the basic functional groups.

Materials and methods

Aminoglycoside analyte solutions (0.15-0.73 M aminoglycoside in 99.97% D_2O) were prepared at a ~10 mg/mL concentration. NMR spectra including: ^{1}H, ^{13}C, HSQC, HMBC, NOESY, and ^{15}N-HMBC were recorded on Bruker Avance III 400 and 500 MHz spectrometers. Trimethylsilylpropanoic acid (TMSP) was used as a reference at 0.00 ppm for ^{1}H and ^{13}C NMR spectroscopies. ^{15}N chemical shift values were measured relative to external CH_3NO_2 set at -511.72 ppm. The pH values were adjusted using 0.5 M NaOD/DCl. MestReNova was used for analysis of the recorded spectra. The nonlinear sigmoidal curve and the inflection point of the sigmoidal curve were determined using GraphPad Prism 7 (Version 2017) after subtraction of 0.5 to convert the measured pD values into pH values (Popov et al., 2006).

Results

The pK_a values of 1-NH_2 and 3-NH_2 of 2-deoxystreptamine are 9.26 and 7.00. The order of ionisation constants for neamine is: N-6' (8.31) > N-1 (7.60) > N-2' (7.11) > N-3 (6.50), for neomycin is: N-6''' (8.76) > N-6' (8.65) > N-1 (8.08) ≈ N-2' (7.98) ≈ N-2''' (8.03) > N-3 (6.86), for tobramycin is: N-6' (9.10) > N-2' (7.75) ≈ N-3'' (7.68) > N-1 (7.55) > N-3 (6.70) and for sisomicin is: N-6' (9.30) > N-3'' (8.50) > N-2' (8.00) > N-1 (7.42) > N-3 (6.22).

Conclusions

^{1}H, ^{13}C, and ^{15}N NMR spectroscopies are powerful techniques when it comes to measuring distinct pK_a values. Also, owing to its sensitivity, ^{1}H NMR spectroscopy requires less time, taking only two minutes for each sample, in comparison to ^{13}C which requires 30 minutes for each sample, and ^{15}N-HMBC, requiring 45 minutes per sample. Unambiguous assignments have been made for each amine on these clinically significant aminoglycoside antibiotics.

Acknowledgements

We thank the Government of the Kingdom of Saudi Arabia for fully funding this studentship.

Reference

Popov, K., Rönkkömäki, H., Lajunen, L.H., 2006. Guidelines for NMR measurements for determination of high and low pKa values (IUPAC Technical Report). Pure and Applied Chem., 78, 663-675.

Individual pK_a values of aminoglycosides determined by different NMR spectroscopies

AbdulAziz H. Alkhzem, Timothy J. Woodman*, and Ian S. Blagbrough*

Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, UK

e-mail: prsisb@bath.ac.uk

The biological activities of these natural product alkaloid aminoglycoside antibiotics depend upon the amino functional group substituents located around the various rings. The ionisation constant (pK_a) is the pH at which functional groups are 50% ionized. Any medication's pK_a values have an important role to play in their physicochemical data and they are also important in the biological activities of drugs. Various NMR reporter nuclei have been used to ascertain the individual pK_a values that cannot be obtained through the use of potentiometric methods, particularly the order in which the functional groups either gain or shed protons. These data have the capacity to enhance comprehension of the order of target mRNA binding of the basic functional groups. The aim is to measure the pK_a values of individual amines on selected aminoglycoside alkaloids from *Streptomyces* and *Micromonospora* by using new combinations of ^{1}H, ^{13}C, and ^{15}N NMR spectroscopic data.

Aminoglycoside analyte solutions (0.15-0.73 M aminoglycoside in 99.97% D_2O) were prepared at a ~10 mg/mL concentration. NMR spectra including ^{1}H, ^{13}C, HSQC, HMBC, NOESY, and ^{15}N-HMBC were recorded on Bruker Avance III 400 and 500 MHz spectrometers. Trimethylsilylpropanoic acid (TMSP) was used as a reference at 0.00 ppm for ^{1}H and ^{13}C NMR spectroscopies. ^{15}N chemical shift values were measured relative to external CH_3NO_2 set at -511.72 ppm. The pH values were adjusted using 0.5 M NaOD/DCl. MestReNova was used for analysis of the recorded spectra. The nonlinear sigmoidal curve and the inflection point of the sigmoidal curve were determined using GraphPad Prism 7 (Version 2017), after subtraction of 0.5 to convert the measured pD values into pH values.[1]

The pK_a values of 1-NH_2 and 3-NH_2 of 2-deoxystreptamine are 9.26 and 7.00. The order of ionisation constants for neamine is: N-6' (8.31) > N-1 (7.60) > N-2' (7.11) > N-3 (6.50), for neomycin is: N-6''' (8.76) > N-6' (8.65) > N-1 (8.08) ≈ N-2' (7.98) ≈ N-2''' (8.03) > N-3 (6.86), for tobramycin is: N-6' (9.10) > N-2' (7.75) ≈ N-3'' (7.68) > N-1 (7.55) > N-3 (6.70) and for sisomicin is: N-6' (9.30) > N-3'' (8.50) > N-2' (8.00) > N-1 (7.42) > N-3 (6.22) (Fig. 1).

In conclusion, ^{1}H, ^{13}C, and ^{15}N NMR spectroscopies are powerful techniques when it comes to measuring distinct pK_a values. Also, owing to of its sensitivity, ^{1}H NMR spectroscopy requires less time, taking only two minutes for each sample, in comparison to ^{13}C which requires 30 minutes for each sample, and ^{15}N-HMBC, requiring 45 minutes per sample. Unambiguous assignments have been made for each amine substituent on these clinically significant aminoglycoside antibiotics.

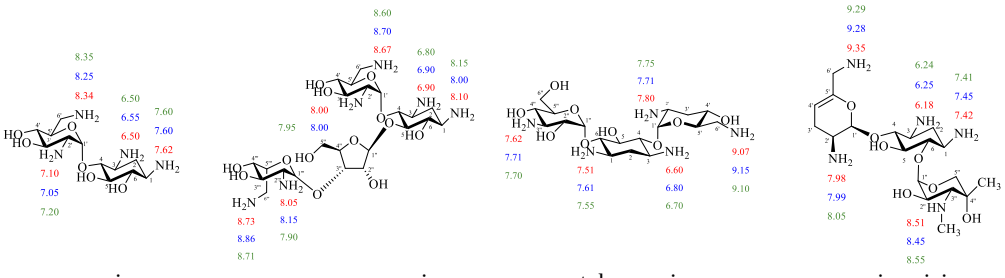


Fig. 1. The pK_a values of each individual amine on aminoglycosides determined using ^{1}H (red), ^{13}C (blue), and ^{15}N-HMBC (black) NMR spectroscopies.

Acknowledgement

We thank the Government of the Kingdom of Saudi Arabia for fully funding this studentship.

Reference[1] Popov, K., Rönkkömäki, H. and Lajunen, L.H., 2006. Guidelines for NMR measurements for determination of high and low pKa values (IUPAC Technical Report). *Pure and Applied Chem.*, *78*, 663-675.

Appendix

Table 4.6a The ^{1}H chemical shifts (ppm) (500 MHz) of H-1/3, H-2ax, H-2eq, H-4/6, and H-5 of 0.245-0.105 M 2-deoxystreptamine were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	H-1/3	H-2ax	H-2eq	H-4/6	H-5
1.78	3.36	1.85	2.48	3.56	3.45
2.94	3.36	1.85	2.48	3.56	3.45
4.28	3.36	1.85	2.48	3.56	3.45
4.85	3.36	1.85	2.47	3.56	3.45
6.42	3.34	1.82	2.48	3.56	3.49
7.08	3.24	1.79	2.38	3.49	3.41
7.94	3.16	1.65	2.34	3.43	3.41
8.52	3.16	1.63	2.32	3.42	3.40
8.75	3.13	1.56	2.29	3.41	3.38
9.19	3.03	1.53	2.27	3.39	3.35
9.68	2.92	1.40	2.11	3.29	3.29
10.01	2.74	1.21	2.00	3.14	3.26
10.84	2.74	1.21	2.00	3.14	3.26

Table 4.6b The ^{1}H chemical shifts (ppm) (500 MHz) of H-1/3, H-2ax, H-2eq, H-4/6, and H-5 of 0.242-0.118 M 2-deoxystreptamine were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	H-1/3	H-2ax	H-2eq	H-4/6	H-5
1.49	3.35	1.85	2.46	3.55	3.42
2.80	3.35	1.84	2.46	3.55	3.42
4.00	3.36	1.84	2.46	3.55	3.42
4.75	3.35	1.84	2.46	3.54	3.42
6.32	3.35	1.82	2.45	3.50	3.39
6.91	3.21	1.78	2.35	3.41	3.39
7.89	3.09	1.58	2.29	3.41	3.35
8.50	3.10	1.55	2.26	3.42	3.32
8.64	3.08	1.52	2.22	3.40	3.30
9.08	2.91	1.40	1.99	3.30	3.32
9.50	2.77	1.30	1.81	3.14	3.23
10.12	2.73	1.25	1.80	3.13	3.21
10.81	2.73	1.25	1.80	3.13	3.21

Table 4.6c The ^{1}H chemical shifts (ppm) (500 MHz) of H-1/3, H-2ax, H-2eq, H-4/6, and H-5 of 0.244-0.110 M 2-deoxystreptamine were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	H-1/3	H-2ax	H-2eq	H-4/6	H-5
1.71	3.37	1.85	2.47	3.55	3.42
2.90	3.36	1.85	2.47	3.55	3.42
4.20	3.37	1.84	2.47	3.54	3.42
4.83	3.36	1.84	2.47	3.54	3.42
6.04	3.33	1.81	2.47	3.52	3.40
6.93	3.22	1.79	2.37	3.45	3.40
7.90	3.12	1.49	2.33	3.42	3.40
8.42	3.12	1.50	2.30	3.43	3.37
8.71	3.10	1.50	2.27	3.42	3.36
9.15	3.08	1.44	1.99	3.31	3.34
9.60	2.81	1.42	1.90	3.25	3.26
10.02	2.75	1.20	1.90	3.13	3.24
10.83	2.75	1.20	1.90	3.13	3.24

Table 4.6d The average of Tables 4.2a, b, and c ^{1}H chemical shifts (ppm) (500 MHz) of H-1/3, H-2ax, H-2eq, H-4/6, and H-5 of 0.243-0.111 M 2-deoxystreptamine were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	H-1/3	H-2ax	H-2eq	H-4/6	H-5
1.66 ± 0.15	3.36 ± 0.01	1.85 ± 0.01	2.47 ± 0.01	3.55 ± 0.02	3.43 ± 0.02
2.88 ± 0.07	3.36 ± 0.01	1.85 ± 0.02	2.47 ± 0.01	3.55 ± 0.02	3.43 ± 0.02
4.16 ±0.14	3.36 ± 0.01	1.84 ± 0.02	2.47 ± 0.01	3.55 ± 0.01	3.43 ± 0.02
4.81± 0.05	3.36 ± 0.01	1.84 ± 0.02	2.47 ± 0.01	3.55 ± 0.01	3.43 ± 0.02
6.38 ± 0.20	3.34 ± 0.01	1.83 ± 0.02	2.46 ± 0.02	3.52 ± 0.03	3.42 ± 0.06
6.97 ± 0.09	3.22 ± 0.02	1.79 ± 0.01	2.36 ± 0.02	3.45 ± 0.04	3.40 ± 0.01
7.91 ± 0.03	3.12 ± 0.04	1.60 ± 0.08	2.32 ± 0.03	3.42 ± 0.01	3.38 ± 0.03
8.48 ± 0.05	3.12 ± 0.03	1.55 ± 0.07	2.29 ± 0.03	3.42 ± 0.02	3.37 ± 0.04
8.70 ± 0.06	3.10 ± 0.03	1.52 ± 0.03	2.26 ± 0.04	3.41 ± 0.01	3.35 ± 0.04
9.14 ± 0.06	3.00 ± 0.09	1.45 ± 0.07	1.95 ± 0.10	3.33 ± 0.05	3.33 ± 0.02
9.59 ± 0.09	2.83 ± 0.08	1.37 ± 0.06	1.94 ± 0.10	3.14 ± 0.08	3.26 ± 0.03
10.05 ± 0.06	2.74 ± 0.01	1.22 ± 0.03	1.94 ± 0.10	3.13 ± 0.02	3.24 ± 0.03
10.83 ± 0.02	2.74 ± 0.01	1.22 ± 0.03	1.91 ± 0.10	3.13 ± 0.02	3.24 ± 0.03

Table 4.7a The ^{13}C chemical shifts (ppm) (125.77 MHz) of C-1/3, C-2, C-4/6, and C-5 of 0.245-0.105 M 2-deoxystreptamine were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	C-1/3	C-2	C-4/6	C-5
1.78	52.88	30.93	75.07	77.44
2.94	52.88	30.93	75.07	77.44
4.28	52.88	30.93	75.07	77.44
4.85	52.88	30.93	75.07	77.44
6.42	52.98	31.03	75.17	77.54
7.08	53.17	33.72	77.00	77.85
7.94	53.24	34.75	77.69	77.98
8.52	53.26	35.31	78.09	78.09
8.75	53.28	36.04	78.62	78.16
9.19	53.30	37.26	79.47	78.32
9.68	53.31	38.20	80.18	78.46
10.01	53.32	38.83	80.84	78.53
10.84	53.33	38.83	80.85	78.53

Table 4.7b The ^{13}C chemical shifts (ppm) (125.77 MHz) of C-1/3, C-2, C-4/6, and C-5 of 0.242-0.118 M 2-deoxystreptamine were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	C-1/3	C-2	C-4/6	C-5
1.49	53.00	31.14	75.20	77.60
2.80	53.00	31.14	75.22	77.61
4.00	53.00	31.05	75.15	77.50
4.75	53.00	31.05	75.15	77.51
6.32	53.10	30.99	75.22	77.55
6.91	52.94	31.81	75.55	77.68
7.89	53.10	34.80	77.64	77.60
8.50	53.18	37.52	79.20	78.30
8.64	53.20	39.77	81.10	78.55
9.08	53.19	39.00	80.02	78.60
9.50	53.28	39.00	79.95	78.66
10.12	53.29	38.81	79.77	78.63
10.81	53.29	38.81	79.77	78.63

Table 4.7c The ^{13}C chemical shifts (ppm) (125.77 MHz) of C-1/3, C-2, C-4/6, and C-5 of 0.244-0.110 M 2-deoxystreptamine were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	C-1/3	C-2	C-4/6	C-5
1.71	52.97	31.20	75.39	77.76
2.90	52.97	31.19	75.41	77.76
4.20	52.97	31.04	75.25	77.68
4.83	52.97	31.04	75.25	77.64
6.04	52.83	31.10	75.84	77.59
6.93	52.95	32.00	76.02	77.68
7.90	53.17	34.88	77.95	77.70
8.42	53.22	37.93	80.43	78.48
8.71	53.20	40.11	81.72	79.09
9.15	53.20	40.00	82.18	78.73
9.60	53.37	39.13	81.70	78.86
10.02	53.35	38.81	81.22	78.64
10.83	53.35	38.81	81.22	78.64

Table 4.7d The average of Tables 4.3a, b, and c ^{13}C chemical shifts (ppm) (125.77 MHz) of C-1/3, C-2, C-4/6, and C-5 of 0.243-0.111 M 2-deoxystreptamine were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	C-1/3	C-2	C-4/6	C-5
1.66 ± 0.15	52.95 ± 0.06	31.09 ± 0.14	75.21 ± 0.16	77.60 ± 0.16
2.88 ± 0.07	52.95 ± 0.06	31.10 ± 0.14	75.23 ± 0.17	77.60 ± 0.16
4.16 ± 0.14	52.95 ± 0.06	31.04 ± 0.07	75.16 ± 0.09	77.54 ± 0.12
4.81 ± 0.05	52.95 ± 0.06	31.04 ± 0.07	75.16 ± 0.09	77.53 ± 0.10
6.38 ± 0.20	52.97 ± 0.14	31.04 ± 0.06	75.41 ± 0.37	77.56 ± 0.03
6.97 ± 0.09	53.02 ± 0.13	32.51 ± 1.05	76.19 ± 0.74	77.74 ± 0.10
7.91 ± 0.03	53.17 ± 0.07	34.81 ± 0.07	77.76 ± 0.17	77.76 ± 0.20
8.48 ± 0.05	53.22 ± 0.04	36.93 ± 1.41	79.24 ± 1.17	78.29 ± 0.20
8.70 ± 0.06	53.23 ± 0.05	38.64 ± 1.26	80.48 ± 1.64	78.60 ± 0.47
9.14 ± 0.06	53.23 ± 0.06	38.76 ± 1.39	80.55 ± 1.43	78.55 ± 0.21
9.58 ± 0.09	53.32 ± 0.05	38.82 ± 0.50	80.61 ± 0.95	78.60 ± 0.20
10.05 ± 0.06	53.32 ± 0.03	38.82 ± 0.01	80.61 ± 0.75	78.60 ± 0.06
10.83 ± 0.02	53.32 ± 0.03	38.82 ± 0.01	80.61 ± 0.75	78.60 ± 0.06

Table 4.8a The ^{15}N HMBC chemical shifts (ppm) (50.67 MHz) of N-1/3 of 0.633-0.370 M 2-deoxystreptamine were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C at different pHs

pH	N-1/3
1.53	48.17
3.75	48.16
4.57	48.17
5.50	48.10
6.01	47.86
6.52	47.22
7.03	46.07
7.66	44.18
8.23	44.24
8.61	42.74
9.27	41.42
10.11	40.81
11.21	40.45
11.84	40.45

Table 4.8b The ^{15}N HMBC chemical shifts (ppm) (50.67 MHz) of N-1/3 of 0.631-0.369 M 2-deoxystreptamine were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C at different pHs

pH	N-1/3
1.55	48.17
3.77	48.18
4.61	48.17
5.57	48.06
5.91	47.71
6.61	47.02
7.11	46.04
7.81	44.52
7.94	44.63
8.64	42.96
9.55	41.42
9.88	40.84
11.58	40.44
11.97	40.44

Table 4.8c The ^{15}N HMBC chemical shifts (ppm) (50.67 MHz) of N-1/3 of 0.630-0.368 M 2-deoxystreptamine were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C at different pHs

pH	**N-1/3**
1.57	48.18
3.73	48.18
4.45	48.18
5.55	48.11
6.21	47.84
6.61	47.01
7.01	46.10
7.69	44.53
7.92	44.33
8.79	43.01
9.17	41.51
10.01	40.87
11.11	40.46
11.41	40.46

Table 4.8d The average of Tables 4.4a, b, and c ^{15}N HMBC chemical shifts (ppm) (50.67 MHz) of N-1/3 of 0.631-0.369 M 2-deoxystreptamine were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C at different pHs

pH	N-1/3
1.55 ± 0.02	48.17 ± 0.01
3.75 ± 0.02	48.17 ± 0.01
4.54 ± 0.08	48.17 ± 0.01
5.54 ± 0.04	48.09 ± 0.03
6.04 ± 0.15	47.80 ± 0.08
6.58 ± 0.05	47.08 ± 0.12
7.05 ± 0.05	46.02 ± 0.03
7.72 ± 0.08	44.41 ± 0.20
8.03 ± 0.17	44.40 ± 0.20
8.68 ± 0.10	42.90 ± 0.14
9.33 ± 0.20	41.45 ± 0.05
10.00 ± 0.12	40.84 ± 0.03
11.30 ± 0.20	40.45 ± 0.01
11.74 ± 0.29	40.45 ± 0.01

Table 4.10 The ^{1}H chemical shifts (ppm) (500 MHz) of H-1, H-3, H-2', H-6'a, and H-6'b of 0.243-0.155 M neamine were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	H-1	H-3	H-2'	H-6'a	H-6'b
3.10 ± 0.10	3.37 ± 0.03	3.55 ± 0.04	3.46 ± 0.03	3.31 ± 0.02	3.49 ± 0.04
3.99 ± 0.08	3.37 ± 0.03	3.55 ± 0.04	3.46 ± 0.03	3.31 ± 0.02	3.49 ± 0.04
4.50 ± 0.06	3.37 ± 0.03	3.55 ± 0.04	3.46 ± 0.03	3.31 ± 0.03	3.49 ± 0.03
5.06 ± 0.06	3.36 ± 0.04	3.52 ± 0.04	3.46 ± 0.03	3.31 ± 0.02	3.49 ± 0.04
5.47 ± 0.07	3.34 ± 0.05	3.46 ± 0.06	3.44 ± 0.05	3.29 ± 0.04	3.48 ± 0.06
5.73 ± 0.08	3.31 ± 0.06	3.37 ± 0.06	3.41 ± 0.05	3.28 ± 0.04	3.47 ± 0.05
6.03 ± 0.10	3.30 ± 0.06	3.31 ± 0.05	3.39 ± 0.06	3.27 ± 0.05	3.46 ± 0.05
6.45 ± 0.15	3.25 ± 0.05	3.19 ± 0.04	3.30 ± 0.05	3.24 ± 0.05	3.45 ± 0.06
7.01 ± 0.09	3.20 ± 0.05	3.12 ± 0.05	3.16 ± 0.06	3.22 ± 0.04	3.44 ± 0.06
7.62 ± 0.08	3.12 ± 0.04	3.05 ± 0.05	3.00 ± 0.06	3.18 ± 0.06	3.40 ± 0.04
8.04 ± 0.09	2.99 ± 0.04	3.00 ± 0.04	2.91 ± 0.05	3.13 ± 0.05	3.34 ± 0.06
8.55 ± 0.07	2.90 ± 0.05	2.96 ± 0.06	2.86 ± 0.06	3.04 ± 0.05	3.25 ± 0.06
8.67 ± 0.06	2.85 ± 0.03	2.94 ± 0.04	2.84 ± 0.05	2.99 ± 0.05	3.21 ± 0.06
8.91 ± 0.05	2.80 ± 0.03	2.91 ± 0.05	2.83 ± 0.04	2.93 ± 0.05	3.15 ± 0.04
9.26 ± 0.04	2.75 ± 0.05	2.89 ± 0.05	2.82 ± 0.03	2.89 ± 0.04	3.07 ± 0.05
9.45 ± 0.06	2.72 ± 0.03	2.87 ± 0.03	2.81 ± 0.02	2.84 ± 0.03	3.05 ± 0.05
9.60 ± 0.10	2.72 ± 0.03	2.87 ± 0.03	2.81 ± 0.02	2.82 ± 0.02	3.04 ± 0.03
10.67 ± 0.08	2.72 ± 0.03	2.87 ± 0.03	2.81 ± 0.02	2.82 ± 0.02	3.04 ± 0.03
12.05 ± 0.09	2.72 ± 0.03	2.86 ± 0.03	2.80 ± 0.02	2.82 ± 0.02	3.03 ± 0.03

Table 4.11 The ^{13}C chemical shifts (ppm) (125.77 MHz) of C-1, C-3, C-2', and C-6' of 0.243-0.155 M neamine were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	C-1	C-3	C-2'	C-6'
3.10 ± 0.10	52.52 ± 0.02	51.08 ± 0.03	56.25 ± 0.04	42.78 ± 0.04
3.99 ± 0.08	52.52 ± 0.02	51.08 ± 0.03	56.22 ± 0.03	42.78 ± 0.03
4.50 ± 0.06	52.52 ± 0.03	51.08 ± 0.03	56.22 ± 0.03	42.78 ± 0.04
5.06 ± 0.06	52.52 ± 0.02	51.08 ± 0.02	56.25 ± 0.04	42.78 ± 0.03
5.47 ± 0.07	52.54 ± 0.04	51.08 ± 0.04	56.35 ± 0.06	42.82 ± 0.05
5.73 ± 0.08	52.55 ± 0.04	51.15 ± 0.04	56.43 ± 0.05	42.93 ± 0.06
6.03 ± 0.10	52.57 ± 0.05	51.20 ± 0.05	56.53 ± 0.05	42.96 ± 0.05
6.45 ± 0.15	52.59 ± 0.05	51.55 ± 0.06	56.72 ± 0.06	43.02 ± 0.04
7.01 ± 0.09	52.81 ± 0.04	52.09 ± 0.03	57.32 ± 0.06	43.31 ± 0.07
7.62 ± 0.08	52.89 ± 0.06	52.18 ± 0.06	57.52 ± 0.04	43.44 ± 0.05
8.04 ± 0.09	53.11 ± 0.05	52.18 ± 0.05	57.74 ± 0.06	43.73 ± 0.04
8.55 ± 0.07	53.13± 0.05	52.18 ± 0.07	57.78 ± 0.06	43.7 9± 0.04
8.67 ± 0.06	53.13 ± 0.05	52.19 ± 0.05	57.83 ± 0.07	44.01 ± 0.04
8.91 ± 0.05	53.13 ± 0.05	52.19 ± 0.05	57.95 ± 0.04	44.14 ± 0.05
9.26 ± 0.04	53.13 ± 0.04	52.19 ± 0.04	58.00 ± 0.04	44.38 ± 0.06
9.45 ± 0.06	53.13 ± 0.03	52.19 ± 0.03	58.01 ± 0.03	44.41 ± 0.04
9.60 ± 0.10	53.13 ± 0.02	52.19 ± 0.04	58.04 ± 0.03	44.40 ± 0.04
10.67 ± 0.08	53.13 ± 0.02	52.19 ± 0.04	58.04 ± 0.03	44.40 ± 0.04
12.05 ± 0.09	53.13 ± 0.02	52.18 ± 0.04	58.04 ± 0.03	44.40 ± 0.04

Table 4.12 The ^{15}N HMBC chemical shifts (ppm) (50.67 MHz) of N-1, N-3, N-2', and N-6' of 0.243-0.155 M neamine were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C at different pHs

pH	N-1	N-3	N-2'	N-6'
3.07 ± 0.015	48.01 ± 0.06	49.05 ± 0.04	43.91 ± 0.04	36.24 ± 0.07
4.04 ± 0.09	48.04 ± 0.05	49.07 ± 0.03	43.91 ± 0.04	36.25 ± 0.07
5.03 ± 0.15	47.92 ± 0.05	49.07 ± 0.08	43.82 ± 0.05	36.25 ± 0.06
5.82 ± 0.10	47.73 ± 0.04	47.1 5 ± 0.06	42.99 ± 0.06	36.11 ± 0.08
7.00 ± 0.15	45.22 ± 0.04	43.51 ± 0.07	39.03 ± 0.15	33.89 ± 0.08
8.03 ± 0.09	43.11 ± 0.15	42.55 ± 0.09	33.04 ± 0.07	31.02 ± 0.09
8.64 ± 0.10	41.55 ± 0.04	42.05 ± 0.02	32.01 ± 0.08	29.92 ± 0.08
9.15 ± 0.15	40.73 ± 0.06	41.51 ± 0.04	31.82 ± 0.07	28.78 ± 0.10
9.80 ± 0.10	40.18 ± 0.04	41.42 ± 0.07	31.56 ± 0.07	26.75 ± 0.04
10.52 ± 0.08	40.1 5± 0.05	41.31 ± 0.06	31.22 ± 0.04	25.65 ± 0.04
11.43 ± 0.08	40.11 ± 0.06	41.28 ± 0.06	31.19 ± 0.04	25.52 ± 0.05

Table 4.14 The ^{1}H chemical shifts (ppm) (500 MHz) of H-1, H-3, H-2', H-6'a, H-6'b, H-2''', H-6'''a, and H-6'''b of 0.218-0.122 M neomycin C were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	H-1	H-3	H-2'	H-6'a	H-6'b	H-2'''	H-6'''a	H-6'''b
2.02 ±0.10	3.41 ±0.05	3.59 ±0.01	3.45 ±0.02	3.37 ±0.01	3.49 ±0.02	3.62 ±0.02	3.33 ±0.02	3.44 ±0.01
4.55 ± 0.08	3.41 ±0.04	3.59 ±0.02	3.45 ±0.02	3.37 ± 0.02	3.50 ±0.02	3.62 ±0.01	3.33 ±0.02	3.44 ±0.02
5.51 ±0.07	3.40 ±0.03	3.40 ±0.03	3.45 ±0.01	3.37 ±0.03	3.50 ±0.01	3.62 ±0.02	3.33 ±0.01	3.44 ±0.03
6.00 ±0.08	3.39 ±0.04	3.30 ±0.01	3.44 ±0.04	3.37 ±0.01	3.49 ±0.04	3.62 ±0.03	3.33 ±0.04	3.44 ±0.01
6.64 ±0.17	3.31 ±0.07	3.15 ±0.07	3.40 ±0.09	3.35 ±0.07	3.47 ±0.09	3.61 ±0.04	3.32 ±0.09	3.43 ±0.07
7.14 ±0.08	3.26 ±0.02	3.07 ±0.05	3.33 ±0.05	3.32 ±0.05	3.44 ±0.05	3.52 ±0.06	3.32 ±0.04	3.43 ±0.05
7.61 ±0.10	3.17 ±0.01	2.99 ±0.04	3.19 ±0.07	3.29 ±0.04	3.41 ±0.07	3.39 ±0.05	3.29 ±0.08	3.40 ±0.04
8.21 ±0.15	3.03 ±0.05	2.93 ±0.02	3.00 ±0.04	3.23 ±0.02	3.35 ±0.04	3.22 ±0.04	3.23 ±0.04	3.34 ±0.02
8.91 ±0.11	2.81 ±0.01	2.90 ±0.07	2.81 ±0.05	2.96 ±0.07	3.08 ±0.05	3.07 ±0.02	3.06 ±0.05	3.17 ±0.07
9.84 ±0.18	2.73 ±0.04	2.89 ±0.05	2.74 ±0.04	2.90 ±0.05	3.03 ±0.03	3.03 ±0.05	2.91 ±0.07	3.02 ±0.04
10.73 ±0.09	2.73 ±0.02	2.89 ±0.02	2.74 ±0.01	2.90 ±0.02	3.03 ±0.01	3.03 ±0.02	2.91 ±0.05	3.02 ±0.02
11.80 ±0.03	2.72 ±0.01	2.89 ±0.02	2.74 ±0.02	2.90 ±0.02	3.03 ±0.02	3.03 ±0.01	2.91 ±0.02	3.02 ±0.02

Table 4.15 The ^{13}C chemical shifts (ppm) (125.77 MHz) of C-1, C-3, C-2', C-6', C-2''', and C-6''' of 0.218-0.122 M neomycin C were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	C-1	C-3	C-2'	C-6'	C-2'''	C-6'''
2.02 ±0.10	51.27 ±0.02	52.69 ±0.02	56.43 ±0.02	43.41 ±0.05	53.48 ±0.01	43.19 ±0.01
4.55 ±0.08	51.27 ±0.02	52.69 ±0.01	56.43 ±0.02	43.41 ±0.04	53.49 ±0.02	43.19 ±0.04
5.51 ±0.07	51.32 ±0.01	52.72 ±0.02	56.42 ±0.01	43.41 ±0.03	53.49 ±0.03	43.19 ±0.03
6.00 ±0.08	51.35 ±0.04	52.96 ±0.03	56.42 ±0.04	43.42 ±0.04	53.52 ±0.01	43.19 ±0.03
6.64 ±0.17	51.41 ±0.09	53.02 ±0.04	56.54 ±0.09	43.43 ±0.07	53.65 ±0.07	43.19 ±0.04
7.14 ±0.08	51.64 ±0.04	53.03 ±0.06	56.77 ±0.05	43.43 ±0.02	53.75 ±0.05	43.20 ±0.06
7.61 ±0.10	51.94 ±0.08	53.03 ±0.05	57.07 ±0.07	43.43 ±0.01	54.18 ±0.04	43.21 ±0.09
8.21 ±0.15	52.61 ±0.04	53.04 ±0.04	57.91 ±0.04	43.69 ±0.05	54.54 ±0.02	43.39 ±0.04
8.91 ±0.11	52.94 ±0.05	53.04 ±0.02	58.21 ±0.05	43.99 ±0.01	55.15 ±0.07	43.74 ±0.08
9.84 ±0.18	53.08 ±0.07	53.04 ±0.05	58.26 ±0.03	44.38 ±0.04	55.39 ±0.05	44.18 ±0.04
10.73 ±0.09	53.08 ±0.05	53.04 ±0.02	58.28 ±0.01	44.59 ±0.02	55.53 ±0.02	44.39 ±0.03
11.80 ±0.03	53.08 ±0.02	53.04 ±0.01	58.29 ±0.02	44.59 ±0.01	55.52 ±0.02	44.39 ±0.02

Table 4.16 The ^{15}N HMBC chemical shifts (ppm) (50.67 MHz) of N-1, N-3, N-2', N-6', N-2''', and N-6''' of 0.392-0.218 M neomycin C were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C at different pHs

pH	N-1	N-3	N-2'	N-6'	N-2'''	N-6'''
1.62 ±0.10	47.83 ±0.02	48.22 ±0.03	43.90 ±0.01	35.61 ±0.05	39.11 ±0.01	36.32 ±0.03
2.94 ±0.05	47.83 ±0.04	48.22 ±0.04	43.90 ±0.02	35.61 ±0.04	39.11 ±0.02	36.32 ±0.02
3.73 ±0.07	47.82 ±0.01	48.22 ±0.03	43.91 ±0.03	35.62 ±0.03	39.12 ±0.01	36.32 ±0.02
4.72 ±0.08	47.82 ±0.06	48.21 ±0.04	43.92 ±0.01	35.55 ±0.04	39.13 ±0.02	36.31 ±0.01
5.60 ±0.07	47.79 ±0.09	47.81 ±0.06	43.41 ±0.07	34.91 ±0.07	39.01 ±0.03	36.29 ±0.04
6.83 ±0.08	47.63 ±0.02	43.80 ±0.02	41.41 ±0.05	34.24 ±0.02	36.79 ±0.04	36.18 ±0.09
7.83 ±0.07	46.01 ±0.10	42.91 ±0.70	38.01 ±0.04	33.71 ±0.01	34.99 ±0.06	35.27 ±0.04
8.74 ±0.10	42.71 ±0.04	42.51 ±0.03	34.11 ±0.02	28.11 ±0.05	34.01 ±0.05	34.01 ±0.08
9.65 ±0.20	40.51 ±0.05	42.11 ±0.04	33.51 ±0.07	25.01 ±0.01	31.55 ±0.04	32.71 ±0.04
10.83 ±0.15	39.92 ±0.03	41.79 ±0.04	33.21 ±0.05	24.93 ±0.04	31.22 ±0.02	32.05 ±0.05
11.62 ±0.05	39.92 ±0.01	41.79 ±0.02	33.21 ±0.02	24.91 ±0.02	31.22 ±0.05	32.02 ±0.07
12.00 ±0.03	39.91 ±0.02	41.80 ±0.01	33.21 ±0.02	24.91 ±0.01	31.20 ±0.02	32.01 ±0.05

Table 4.18 The ^{1}H chemical shifts (ppm) (500 MHz) of H-1, H-3, H-2', H-2''', H-6'''a, and H-6'''b of 0.335-0.208 M paromomycin were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	H-1	H-3	H-2'	H-2'''	H-6'''a	H-6'''b
1.51	3.36	3.58	3.45	3.65	3.44	3.44
4.01	3.36	3.56	3.44	3.65	3.43	3.43
4.83	3.37	3.56	3.44	3.65	3.44	3.44
6.01	3.39	3.55	3.44	3.64	3.43	3.43
7.01	3.31	3.31	3.35	3.58	3.41	3.41
7.95	3.13	3.07	3.11	3.35	3.36	3.36
8.57	2.96	2.97	2.91	3.16	3.27	3.27
9.75	2.73	2.88	2.75	3.03	2.94	3.04
11.25	2.73	2.86	2.72	3.01	2.82	2.93

Table 4.19 The ^{13}C chemical shifts (ppm) (125.77 MHz) of C-1, C-3, C-2', C-2''', and C-6''' of 0.335-0.208 M paromomycin were measured relative to TMSP in 99.97% D_2O at 25°C

pH	C-1	C-3	C-2'	C-2'''	C-6'''
1.51	49.67	48.78	53.78	50.63	40.41
4.01	49.68	48.91	53.78	50.63	40.41
4.83	49.91	48.85	53.99	50.71	40.49
6.01	50.21	48.85	53.99	50.77	40.44
7.01	50.43	51.21	54.72	51.46	40.44
7.95	50.81	52.77	56.28	52.11	41.09
8.57	52.81	53.01	57.81	54.79	41.44
9.75	52.93	52.93	58.15	55.31	43.69
11.25	52.93	53.02	58.19	55.42	44.06

Table 4.20 The ^{15}N HMBC chemical shifts (ppm) (50.67 MHz) of N-1, N-3, N-2', N-2‴, and N-6‴ of 0.335-0.208 M paromomycin were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C at different pHs

pH	N-1	N-3	N-2'	N-2‴	N-6‴
1.51	47.31	49.21	43.01	38.34	37.17
4.01	47.31	49.21	43.01	38.33	37.17
4.83	47.31	49.21	43.01	38.33	37.16
6.01	47.14	47.81	43.41	38.69	35.98
7.01	45.15	46.55	42.09	37.34	36.05
7.95	45.15	41.28	38.49	32.39	35.51
8.57	42.89	40.66	35.24	28.01	33.81
9.75	42.01	40.19	32.64	25.19	25.33
11.25	42.27	39.42	32.64	25.01	25.16

Table 4.22 The ^{1}H chemical shifts (ppm) (500 MHz) of H-1, H-3, H-2', H-6'a, H-6'b, and H-3" of 0.251-0.132 M tobramycin were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	H-1	H-3	H-2'	H-6'a	H-6'b	H-3''
2.61 ± 0.09	3.65 ± 0.01	3.60 ± 0.01	3.69 ± 0.01	3.32 ± 0.01	3.45 ± 0.01	3.52 ± 0.02
4.09 ± 0.10	3.65 ± 0.02	3.60 ± 0.02	3.69 ± 0.02	3.33 ± 0.02	3.46 ± 0.02	3.53 ± 0.03
4.50 ± 0.07	3.65 ± 0.03	3.60 ± 0.01	3.69 ± 0.03	3.33 ± 0.03	3.46 ± 0.03	3.53 ± 0.09
5.85 ± 0.08	3.60 ± 0.04	3.48 ± 0.02	3.68 ± 0.01	3.33 ± 0.05	3.47 ± 0.07	3.53 ± 0.07
6.37 ± 0.12	3.52 ± 0.07	3.34 ± 0.03	3.64 ± 0.07	3.33 ± 0.07	3.46 ± 0.02	3.50 ± 0.05
7.24 ± 0.09	3.36 ± 0.02	3.16 ± 0.01	3.50 ± 0.05	3.30 ± 0.05	3.44 ± 0.03	3.37 ± 0.04
7.51 ± 0.03	3.31 ± 0.03	3.11 ± 0.02	3.40 ± 0.04	3.31 ± 0.04	3.44 ± 0.09	3.32 ± 0.08
7.81 ± 0.10	3.22 ± 0.04	3.08 ± 0.03	3.31 ± 0.01	3.28 ± 0.01	3.42 ± 0.07	3.20 ± 0.02
8.57 ± 0.03	3.10 ± 0.06	3.02 ± 0.01	3.13 ± 0.02	3.20 ± 0.02	3.34 ± 0.05	3.09 ± 0.03
9.15 ± 0.07	2.98 ± 0.05	2.95 ± 0.02	3.07 ± 0.03	3.08 ± 0.03	3.21 ± 0.04	3.05 ± 0.04
9.53 ± 0.08	2.94 ± 0.04	2.91 ± 0.01	3.01 ± 0.08	2.98 ± 0.07	3.11 ± 0.08	3.02 ± 0.02
10.21 ± 0.05	2.91 ± 0.02	2.88 ± 0.02	2.96 ± 0.07	2.87 ± 0.07	3.00 ± 0.02	3.01 ± 0.02
11.25 ± 0.10	2.90 ± 0.05	2.88 ± 0.03	2.96 ± 0.01	2.84 ± 0.05	2.97 ± 0.03	3.01 ± 0.03
11.95 ± 0.03	2.90 ± 0.02	2.88 ± 0.01	2.95 ± 0.02	2.84 ± 0.04	2.97 ± 0.04	3.00 ± 0.04
12.01 ± 0.07	2.90 ± 0.01	2.88 ± 0.07	2.95 ± 0.03	2.84 ± 0.02	2.98 ± 0.02	3.00 ± 0.02

Table 4.23 The ^{13}C chemical shifts (ppm) (125.77 MHz) of C-1, C-3, C-2', C-6', and C-3'' of 0.251-0.132 M tobramycin were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	C-1	C-3	C-2'	C-6'	C-3''
2.61 ± 0.09	50.61 ± 0.01	52.51 ± 0.02	51.21 ± 0.01	42.91 ± 0.02	56.99 ± 0.02
4.09 ± 0.10	50.59 ± 0.02	52.51 ± 0.03	51.21 ± 0.02	42.91 ± 0.03	56.98 ± 0.05
4.50 ± 0.07	50.58 ± 0.04	52.51 ± 0.09	51.19 ± 0.03	42.89 ± 0.09	56.97 ± 0.02
5.85 ± 0.08	50.71 ± 0.02	52.56 ± 0.02	51.34 ± 0.05	43.01 ± 0.07	56.97 ± 0.01
6.37 ± 0.12	50.84 ± 0.05	52.67 ± 0.03	51.42 ± 0.07	43.04 ± 0.05	56.96 ± 0.10
7.24 ± 0.09	51.11 ± 0.07	52.97 ± 0.09	51.57 ± 0.05	43.05 ± 0.04	57.01 ± 0.03
7.51 ± 0.03	51.19 ± 0.01	53.05 ± 0.07	51.74 ± 0.04	43.09 ± 0.08	57.12 ± 0.03
7.81 ± 0.10	51.39 ± 0.10	53.06 ± 0.05	51.81 ± 0.01	43.11 ± 0.02	57.41 ± 0.07
8.57 ± 0.03	51.55 ± 0.03	53.07 ± 0.04	51.92 ± 0.02	43.34 ± 0.02	57.52 ± 0.05
9.15 ± 0.07	51.63 ± 0.06	53.07 ± 0.08	52.07 ± 0.03	43.65 ± 0.03	57.61 ± 0.02
9.53 ± 0.08	51.77 ± 0.02	53.06 ± 0.02	52.15 ± 0.07	43.92 ± 0.09	57.64 ± 0.01
10.21 ± 0.05	51.94 ± 0.01	53.06 ± 0.03	52.26 ± 0.07	44.32 ± 0.07	57.68 ± 0.10
11.25 ± 0.10	51.97 ± 0.03	53.07 ± 0.04	52.29 ± 0.05	44.38 ± 0.05	57.69 ± 0.03
11.95 ± 0.03	51.95 ± 0.07	53.08 ± 0.02	52.31 ± 0.04	44.43 ± 0.04	57.69 ± 0.03
12.01 ± 0.07	51.96 ± 0.01	53.09 ± 0.02	52.32 ± 0.02	44.44 ± 0.08	57.69 ± 0.07

Table 4.24 The ^{15}N HMBC chemical shifts (ppm) (50.67 MHz) of N-1, N-3, N-2', N-6', and N-3'' of 0.740-0.370 M tobramycin were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C at different pHs

pH	N-1	N-3	N-2'	N-6'	N-3''
1.61 ± 0.09	47.71 ± 0.02	49.27 ± 0.03	48.19 ± 0.03	36.74 ± 0.05	42.83 ± 0.02
2.55 ± 0.13	47.71± 0.05	49.26 ± 0.06	48.19 ± 0.09	36.74 ± 0.04	42.83 ± 0.04
3.85 ± 0.07	47.68 ± 0.02	49.22 ± 0.02	48.20 ± 0.07	36.75 ± 0.01	42.82 ± 0.06
5.07 ± 0.08	47.20 ± 0.01	48.81 ± 0.02	48.20 ± 0.05	36.61 ± 0.05	42.51 ± 0.07
5.71 ± 0.12	46.91 ± 0.10	48.06 ± 0.07	48.13 ± 0.03	36.44 ± 0.04	42.38 ± 0.10
6.02 ± 0.18	46.51 ± 0.03	47.51 ± 0.06	47.71 ± 0.09	36.31 ± 0.01	42.35 ± 0.12
7.03 ± 0.09	44.51 ± 0.03	44.43 ± 0.02	44.94 ± 0.03	35.53 ± 0.02	40.54 ± 0.05
7.75 ± 0.17	43.01 ± 0.02	43.59 ± 0.04	43.69 ± 0.03	34.97 ± 0.03	38.01 ± 0.05
8.38 ± 0.08	42.05 ± 0.02	42.81 ± 0.03	42.42 ± 0.02	33.68 ± 0.07	36.43 ± 0.02
8.71 ± 0.12	41.72 ± 0.05	42.55 ± 0.07	41.71 ± 0.02	32.82 ± 0.05	35.53 ± 0.03
9.50 ± 0.05	40.83 ± 0.02	41.87 ± 0.01	40.03 ± 0.05	27.35 ± 0.04	33.97 ± 0.01
10.09 ± 0.07	40.55 ± 0.01	41.42 ± 0.02	39.42 ± 0.02	24.91 ± 0.01	33.47 ± 0.01
11.34 ± 0.10	40.21 ± 0.10	41.21 ± 0.04	39.41 ± 0.01	23.71 ± 0.02	33.31 ± 0.03
11.94 ± 0.08	40.19 ± 0.03	41.11 ± 1.02	39.40 ± 0.08	23.70 ± 0.03	33.29 ± 0.02

Table 4.26 The ^{1}H chemical shifts (ppm) (500 MHz) of H-1, H-3, H-2', H-6'a, H-6'b and H-3" of 1.315-0.822 M kanamycin B were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	H-1	H-3	H-2'	H-6'a	H-6'b	H-3''
1.51	3.59	3.66	3.53	3.53	3.26	3.44
4.92	3.59	3.66	3.53	3.53	3.26	3.44
5.54	3.55	3.66	3.50	3.54	3.28	3.41
6.54	3.41	3.35	3.39	3.51	3.25	3.31
7.01	3.45	3.19	3.25	3.48	3.25	3.27
8.27	3.17	2.89	3.01	3.38	3.16	3.03
9.05	2.97	2.89	2.91	3.19	2.99	2.79
10.51	2.91	2.89	2.84	2.99	2.89	2.75
11.10	2.87	2.88	2.81	2.99	2.81	2.75
11.51	2.87	2.88	2.81	2.99	2.81	2.75

Table 4.27 The ^{13}C chemical shifts (ppm) (125.77 MHz) of C-1, C-3, C-2', C-6', and C-3" of 1.315-0.822 M kanamycin B were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	C-1	C-3	C-2'	C-6'	C-3''
1.51	51.06	52.28	57.58	43.14	56.33
4.92	51.09	52.28	57.59	43.16	56.34
5.54	51.04	52.29	57.57	43.16	56.32
6.54	51.27	52.31	57.67	43.21	56.57
7.01	51.31	52.49	57.8	43.22	56.56
8.27	52.51	52.55	58.2	43.23	57.57
9.05	53.49	52.55	58.29	43.74	57.81
10.51	53.51	52.58	58.31	44.41	57.99
11.10	53.55	52.58	58.32	44.51	58.01
11.51	53.56	52.59	58.34	44.52	58.09

Table 4.28 The ^{15}N HMBC chemical shifts (ppm) (50.67 MHz) of N-1, N-3, N-2', N-6', and N-3" of 1.315-0.822 M kanamycin B were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C at different pHs

pH	N-1	N-3	N-2'	N-6'	N-3''
1.51	47.21	48.22	43.14	36.01	42.43
4.92	47.22	48.25	43.14	35.01	42.47
5.54	46.77	47.19	42.69	35.72	42.52
6.54	46.32	46.32	40.61	35.66	42.04
7.01	45.57	44.85	37.81	35.51	41.37
8.27	43.33	43.63	34.15	35.17	35.61
9.05	41.33	42.44	34.03	29.72	34.47
10.51	41.25	42.35	31.74	28.55	33.99
11.10	41.03	42.09	31.51	27.68	33.82
11.51	41.03	42.09	31.51	27.68	33.82

Table 4.30 The ^{1}H chemical shifts (ppm) (500 MHz) of H-1, H-3, H-2', H-6'a, H-6'b, and H-3" of 0.506-0.434 M netilmicin were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	H-1	H-3	H-2'	H-6'a	H-6'b	H-3''
2.05	3.57	3.55	3.93	3.34	3.14	3.52
5.18	3.56	3.55	3.92	3.35	3.13	3.51
6.77	3.47	3.11	3.84	3.31	3.09	3.49
7.52	3.35	2.88	3.75	3.30	3.05	3.39
8.11	3.18	2.81	3.63	3.20	2.95	3.21
9.51	2.76	2.76	3.34	3.01	2.77	2.59
10.41	2.74	2.73	3.33	2.71	2.45	2.57
11.51	2.74	2.71	3.32	2.71	2.44	2.52

Table 4.31 The ^{13}C chemical shifts (ppm) (125.77 MHz) of C-1, C-3, C-2', C-6', and C-3'' of 0.506-0.434 M netilmicin were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	C-1	C-3	C-2'	C-6'	C-3''
2.05	58.74	50.73	85.32	43.09	66.33
5.18	58.8	50.71	85.64	43.08	66.36
6.77	59.09	51.58	86.02	43.10	66.37
7.52	59.23	52.05	86.53	43.23	66.39
8.11	59.58	52.05	87.01	43.30	66.41
9.51	60.19	52.01	88.38	43.51	66.52
10.41	60.25	52.05	88.44	43.83	66.56
11.51	60.25	52.13	88.45	43.86	66.57

Table 4.32 The ^{15}N HMBC chemical shifts (ppm) (50.67 MHz) of N-1, N-3, N-2', N-6', and N-3'' of 0.506-0.434 M netilmicin were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C at different pHs

pH	N-1	N-3	N-2'	N-6'	N-3''
2.05	59.81	47.87	42.25	39.50	42.61
5.18	59.81	47.82	42.25	39.45	42.57
6.77	59.44	42.81	42.22	39.82	42.51
7.52	59.31	41.01	40.78	38.77	41.71
8.11	58.77	40.83	40.09	38.89	39.51
9.51	57.42	40.31	31.82	33.33	31.82
10.41	57.35	40.03	31.41	28.81	31.35
11.51	57.32	39.92	31.22	28.71	31.29

Table 4.34 The ^{1}H chemical shifts (ppm) (500 MHz) of H-1, H-3, H-2', H-6'a, H-6'b, and H-3" of 0.083-0.063 M sisomicin were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	H-1	H-3	H-2'	H-6'a	H-6'b	H-3''
2.35	3.56	3.58	3.95	3.71	3.71	3.51
4.12	3.56	3.58	3.95	3.71	3.71	3.51
5.18	3.56	3.56	3.95	3.71	3.71	3.51
5.56	3.56	3.50	3.95	3.71	3.71	3.51
5.92	3.54	3.41	3.95	3.70	3.70	3.50
6.51	3.47	3.13	3.91	3.70	3.70	3.48
7.11	3.34	3.05	3.82	3.68	3.68	3.43
7.51	3.19	3.00	3.73	3.66	3.66	3.39
8.06	3.08	2.98	3.42	3.63	3.63	3.21
8.51	2.99	2.95	3.34	3.58	3.58	3.05
9.01	2.89	2.93	3.22	3.50	3.50	2.83
9.57	2.81	2.91	3.13	3.29	3.29	2.65
10.01	2.78	2.90	3.09	3.21	3.21	2.61
10.65	2.76	2.90	3.07	3.15	3.15	2.57
11.31	2.76	2.90	3.07	3.14	3.14	2.57
12.11	2.76	2.90	3.07	3.14	3.14	2.57

Table 4.35 The ^{13}C chemical shifts (ppm) (125.77 MHz) of C-1, C-3, C-2', C-6', and C-3" of 0.083-0.063 M sisomicin were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	C-1	C-3	C-2'	C-6'	C-3''
2.35	50.99	52.71	48.76	43.38	66.16
4.12	51.01	52.71	48.76	43.38	66.16
5.18	51.01	52.75	48.76	43.39	66.17
5.56	51.01	52.95	48.76	43.39	66.18
5.92	51.03	53.15	48.77	43.41	66.19
6.51	51.06	53.30	48.80	43.42	66.29
7.11	51.15	53.41	48.85	43.41	66.36
7.51	51.23	53.48	48.94	43.45	66.41
8.06	51.69	53.54	49.08	43.56	66.48
8.51	51.85	53.59	49.16	43.81	66.51
9.01	51.93	53.63	49.19	44.21	66.58
9.57	52.09	53.68	49.25	44.65	66.69
10.01	52.11	53.68	49.29	44.98	66.77
10.65	52.16	53.69	49.35	45.20	66.88
11.31	52.14	53.69	49.38	45.25	66.88
12.11	52.14	53.69	49.38	45.25	66.88

Table 4.36 The ^{15}N HMBC chemical shifts (ppm) (50.67 MHz) of N-1, N-3, N-2', N-6', and N-3" of 0.160-0.112 M sisomicin were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C at different pHs

pH	N-1	N-3	N-2'	N-6'	N-3''
2.00	46.99	47.49	44.41	40.24	42.47
3.48	46.98	47.47	44.41	40.24	42.46
4.11	46.97	47.20	44.37	40.23	42.62
5.05	46.76	46.51	43.18	39.73	42.37
5.90	46.64	45.15	42.70	39.67	42.30
7.20	44.69	42.52	41.99	40.11	42.12
7.80	42.12	41.80	40.61	39.61	39.05
8.91	40.51	41.01	35.12	37.21	35.39
9.55	40.38	41.82	34.01	33.10	32.83
10.52	40.53	41.16	32.80	29.47	31.07
11.21	40.49	41.15	32.77	29.46	31.05
12.05	40.48	41.15	32.77	29.47	31.05

Table 4.38 The ^{1}H chemical shifts (ppm) (500 MHz) of H-3, H-6'a, H-6'b, H-3", H-4'"a and H-4'"b of 0.896-0.597 M amikacin were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	H-3	H-6'a	H-6'b	H-3''	H-4'''a	H-4'''b
3.91	3.58	3.49	3.21	3.43	3.22	3.22
5.58	3.57	3.49	3.19	3.42	3.19	3.19
6.51	3.52	3.48	3.19	3.42	3.19	3.19
7.03	3.47	3.47	3.18	3.38	3.17	3.17
7.63	3.27	3.44	3.18	3.31	3.18	3.18
8.13	3.09	3.38	3.13	3.16	3.18	3.18
9.61	2.95	3.07	2.84	2.98	3.03	3.03
10.08	2.94	3.01	2.81	2.98	2.92	2.92
11.37	2.93	2.98	2.76	2.98	2.74	2.74
12.02	2.91	2.98	2.75	2.96	2.71	2.71

Table 4.39 The ^{13}C chemical shifts (ppm) (125.77 MHz) of C-3, C-6', C-3", and C-4"' of 0.896-0.597 M amikacin were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	C-3	C-6'	C-3''	C-4'''
3.91	50.67	42.01	56.7	39.51
5.58	50.68	42.01	56.71	39.51
6.51	50.71	42.09	56.76	39.51
7.03	50.83	42.22	56.88	39.52
7.63	50.99	42.44	57.18	39.55
8.13	51.07	42.74	57.45	39.58
9.61	51.31	43.76	57.81	39.77
10.08	51.37	44.26	57.85	39.88
11.37	51.37	44.43	57.91	40.09
12.02	51.37	44.44	57.91	40.11

Table 4.40 The ^{15}N HMBC chemical shifts (ppm) (50.67 MHz) of N-3, N-6', N-3", and N-4"' of 0.896-0.597 M amikacin were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C at different pHs

pH	N-3	N -6'	N -3''	N -4'''
3.91	47.66	35.64	42.56	41.81
5.58	47.81	35.63	42.51	41.51
6.51	47.12	35.72	42.01	41.75
7.03	46.73	35.63	41.84	41.48
7.63	44.31	35.11	40.41	41.51
8.13	42.69	34.02	37.73	41.43
9.61	41.41	25.01	33.82	38.81
10.08	41.65	24.53	33.72	36.49
11.37	41.07	23.82	33.58	32.01
12.02	41.06	23.71	33.71	32.01

Table 4.42 The ^{1}H chemical shifts (ppm) (500 MHz) of H-1, H-3 and H-2' of 0.738-0.527 M streptomycin were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	H-1	H-3	H-2'
4.61	3.62	3.51	3.32
5.71	3.62	3.51	3.32
8.07	3.62	3.51	3.04
10.35	3.62	3.51	2.58
11.15	3.61	3.48	2.57
11.72	3.59	3.44	2.55
12.22	3.55	3.21	2.55
13.51	3.11	3.12	2.55
14.11	2.89	3.12	2.55

Table 4.43 The ^{13}C chemical shifts (ppm) (125.77 MHz) of C-1, C-3 and C-2' of 0.738-0.527 M streptomycin were measured relative to TMSP in 99.97% D_2O at 25°C at different pHs

pH	C-1	C-3	C-2'
4.61	60.55	60.92	63.34
5.71	60.62	61.01	63.41
8.07	60.64	61.01	64.58
10.35	60.7	61.19	65.73
11.15	60.71	61.48	65.81
11.72	60.77	61.88	65.71
12.22	61.11	62.77	65.71
13.51	62.53	63.21	65.71
14.11	63.01	63.27	65.71

Table 4.44 The ^{15}N HMBC chemical shifts (ppm) (50.67 MHz) of N-1, N-3 and N-2' of 0.738-0.527 M streptomycin were measured relative to CH_3NO_2 in 99.97% D_2O at 25°C at different pHs

pH	N-1	N-3	N-2'
4.61	96.83	96.02	44.49
5.71	96.82	96.01	44.41
8.07	96.82	96.01	40.21
10.35	96.82	96.01	33.71
11.15	96.77	95.82	33.82
11.72	96.74	94.99	33.81
12.22	96.41	94.71	33.81
13.51	95.17	93.84	33.81
14.11	94.81	93.23	33.81